[redacted]

SUBJECT: Risk Assessment for TERA R-21-0002

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All exemptions are under (b)(4)

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DATE: May 4, 2021

SUMMARY

The Agency has received a TSCA Experimental Release Application (TERA) from Synthetic Genomics, Inc. (La Jolla, CA) to test three intergeneric eukaryotic algal strains in open ponds at their Synthetic Genomics, Inc. – California Advanced Algae Facility (CAAF) in Calipatria, CA. The subject strains for this risk assessment are [redacted] (R-21-0002.01), [redacted] (R-21-0002.02), and [redacted] (R-21-0002.03).

The submitter plans to field test these intergeneric algal strains over a period of 18 months in open miniponds, 0.1A ponds, and in large ponds up to 1 acre (1,000,000 L) in size. Monitoring will continue for 6 months after the open pond cultivations are terminated. The subject strains were genetically modified by [redacted] and through [redacted] for increased lipid production resulting in lipid productivity many times higher than the wild-type strain. The three subject strains also show promise for scalability in the outdoor environment.

The parental strain, [redacted], was [redacted]. Phylogenetic analysis was performed to assess the taxonomic designation of this parental strain. The sequence aligned in [redacted] however, the sequence did not match with any species within the genus. Thus, the isolate is just referred to as [redacted]

There are [redacted] in this TERA. One is [redacted], which was the recipient for the [redacted]. It was selected following [redacted] the wild-type strain [redacted] The [redacted], [redacted] is another [redacted] from the parental wild-type [redacted]. This latter strain was [redacted], whereas [redacted] was the recipient for [redacted]

The three subject strains were intended to be created by [redacted] However, the [redacted], not just [redacted] However, there is data to support that there is [redacted] in each of the three subject strains because these three strains can [redacted] The [redacted] encoding a [redacted] that contains [redacted] and [redacted]. The subject strains also contain an intergeneric [redacted]

[redacted] which was an unintended result of the [redacted]. It was part of the [redacted] that [redacted] into the strains.

There is low risk of injury to human health and the environment associated with the small-scale field testing of the three intergeneric strains of [redacted] The recipient species does not present concerns for pathogenicity, toxicity, or allergenicity to humans, even to potentially exposed or susceptible subpopulations. The introduced genetic material does not affect the low potential for any adverse human health effects. Although an [redacted] was used as [redacted] and a [redacted] was used for [redacted] and [redacted] are not [redacted] so there is little concern for [redacted] Horizontal gene transfer is expected to be low if not absent in green algae in general.

The small-scale field testing of the [redacted] subject strains is expected to present low risk to the environment since members of the genus [redacted], which are ubiquitous in the environment, do not pose hazards to animals, plants, or other organisms. The introduced genetic material does not affect the low potential of adverse environmental effects. The genetic material does not impart any competitive growth or dispersal advantages as compared to the parental strain. Any time algae are grown in open ponds aerial dispersal is to be expected from wind and from the agitation of the paddle wheels used in the raceway ponds for mixing. Dispersal of the algal cells into the environment is thought to be likely since [redacted] is closely related to (and also previously named) the more widely referenced [redacted], which is an algal species known to be routinely transmitted in air. However, data generated by the company with their extensive monitoring during and after their [redacted] open pond algae field tests using [redacted] showed a low level of dispersal at distance even though some dispersal was observed in trap buckets in close proximity to the ponds.

The parental strain is euryhaline which means it can tolerate a wide range of salinities. Thus, survival may be expected in both fresh and marine waters if the subject strains are dispersed to water bodies. However, the subject strains are not expected to be invasive or out-compete other algae in the environment. Metagenomic data generated from SGI's monitoring in [redacted] demonstrated that [redacted] is not invasive in samples of water obtained from water bodies in the surrounding environment. Therefore, no adverse environmental effects are expected if the strains survive in terrestrial and aquatic environments into which they are disseminated.

Even though there may be dispersal of the three [redacted] subject strains into the environment from the proposed small-scale field testing in open ponds, there is low risk associated with these field tests since the subject strains pose low human health and ecological hazards. Although the use of [redacted] in microorganisms intended for environmental release is typically undesirable, the [redacted] do not pose concerns. [redacted] So there is little concern for [redacted].

The overall purpose of this submission is to assess and monitor [redacted] genetically engineered algae research strains with enhanced lipid production traits in outdoor ponds, at multiple scales, for research and development. This submission builds upon SGI's [redacted] for algal biofuels (R-19-0001, [redacted]). The submitter added that this TERA also aims to continue to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. Gaining insight into how algal strains (top candidates today as well as those to be developed) perform in industrially-relevant

[redacted]

settings will inform the design of the technology and ultimately accelerate its development and deployment. It will also reduce the risk of failure that comes with continuing to design a technology without knowing the conditions and constraints it will ultimately face at scale.

I. INTRODUCTION

EPA has received a TSCA Environmental Release Application (TERA) from Synthetic Genomics, Inc. (SGI, La Jolla, CA) to test three algal constructs, [redacted]), in a field trial in open ponds.

The introduced intergeneric DNA components present in the final constructs [redacted] The [redacted] is used as [redacted] The [redacted] were intended to [redacted] in [redacted] to achieve higher lipid productivity. The incorporation of the [redacted] coming from the [redacted], and serves no function for the submitter. The [redacted], will be used by SGI to specifically track the three subject strains in open-culture and the environment. [redacted] and [redacted] although the genes differed between the strains with some overlap.

The submitter's primary objective for this trial is to [redacted]t and cultivate one to three subject strains at a scale of ≥ 25m^2 and maintain production-like operations for up to 18 months. The maximum total duration of this trial is approximately 24 months which includes 6 months beyond the in-pond cultivation phase for post-cultivation environmental monitoring, analysis, and reporting.

This TERA (R-21-0002) is the [redacted] Previous algae TERAs include [redacted]), R-17-0002 and R-18-0001 (both *Chlorella sorokiniana*), R-19-0001 (*Parachlorella* sp.), and [redacted]

II. TAXONOMY AND CHARACTERIZATION

A. Recipient Microorganisms

The submitter identifies the parental organism as a wild-type [redacted]). This strain was [redacted] was then subjected to [redacted]) to create [redacted]. These recipient strains were selected for their marginally higher biomass and lipid productivity compared to the wild-type strain.

The taxonomic identity of [redacted] was substantiated as belonging to the genus [redacted] using 18S SSU (small subunit) rRNA which is a commonly used phylogenetic marker. Although deemed to be [redacted], the phylogenetic analysis was not able to identify [redacted] at the species level as there was no match to existing species within the genus. Hence the nomenclature [redacted]. is used.

1. The Genus [redacted]

The [redacted] genus was previously assessed by EPA in another TERA submission last year, [redacted] is a genus of green algae that is commonly found in fresh waters through the world although it

[redacted]

is euryhaline meaning it can tolerate a wide range of salinities. The recipient strains grow under a broad range of abiotic conditions with temperature ranges from [redacted], and salinity up to [redacted]

[redacted] is a member of the [redacted] which is a large algal group including [redacted]). [redacted] algae are found mostly in soil and freshwater.

According to Rahman (2021), the taxonomy of the green planktonic genus [redacted] to some extent is unsettled. Previous straight-forward classification schemes for [redacted] were based on its simple morphological structure which, in the absence of other data, appeared definitive. However, increasing availability of molecular genetics data during the last 20 years has changed that apparently simple taxonomic determination into a much more complex system. The use of the word [redacted] as a genus name for phytoplankton dates at least to the 1880s ([redacted]). [redacted], which was initially reported in [redacted], is currently classified as [redacted], the type species for a genus in a family [redacted] that is closely related to the family [redacted] but phylogenetically distinguishable using SSU rRNA and Ribulose-1,5-bisphosphate Carboxylase Oxygenase (RuBisCO) large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) sequence analysis ([redacted]). However, Algaebase reported [redacted] as the correct name for the species [redacted]). This indicates the nature of problems in trying to assign names of [redacted] species with a high degree of accuracy in the current rapidly fluctuating state of taxonomy. In May 2019, [redacted] were then accepted taxonomically. In August 2019, [redacted] were accepted taxonomically. These show the rapidly changing nature of [redacted] even within Algaebase. Difficulties with taxonomic classification for these organisms may also be seen by comparing pictures of the type species with a species now reclassified into another genus (Figure 1).

[redacted]

Table 1 below summarizes the current understanding of the phylogenetic hierarchy for [redacted] as defined by Algaebase. Inclusion of the genus [redacted] in the Class [redacted] and the Family [redacted] has been consistent for many years, despite many changes in genera and species included in those taxonomic classification levels. The number of species formally accepted as within the genus [redacted] has varied substantially even within the last few years [redacted]). Many species also have been consistently listed as part of [redacted], such as [redacted]

Table 1. Phylogenetic relationships for [redacted])

Empire – Eukaryota

[redacted]

Kingdom – Plantae
Subkingdom – Viridiplantae
Infrakingdom – Chlorophyta Infrakingdom
Phylum – Chlorophyta
[redacted]
[redacted]
[redacted]
[redacted]
[redacted]

The difficulties associated with morphology as the basis of taxonomy for [redacted] and related genera was illustrated in a 2007 publication [redacted]). The author considered various morphological features such as presence of a mucilage envelope, autospore release features, chloroplast number, presence or absence of pyrenoids, cell shape, presence or absence of thickening at cell poles, cell wall structure, asymmetry of cell formations, cell size, and position of cells in colonies in order to determine taxonomy. Analysis of taxonomic morphology required [redacted] sketches and approximately [redacted] literature citations. The authors acknowledged that much more work remained to be done preferably with strains isolated from various regions of the world [redacted]). The analysis is also complicated by the problem that many morphological features are variable and function as soft measures for classification, since environmental conditions have been shown to alter many of them [redacted]).

2. The species [redacted]

As mentioned earlier, although 18S SSU rRNA was able to correctly place the parent microorganism in the genus [redacted] by comparing with other green algae (order [redacted]) (Figure 2), it was not able to identify it at the species level, resulting in [redacted] being used in the submission.

[redacted]

According to the TERA and Rahman (2021), the wild-type [redacted], along with the recipient and subject strains grow typically as [redacted]; however, a broader range of sized [redacted] were observed (Figure 3). The submitter also notes that while [redacted] are common, so are [redacted] containing [redacted]-

[redacted]. These observations are phenotypically and morphologically consistent with an [redacted] assignment.

[redacted]

B. Donor Microorganisms

1. [redacted]

The [redacted] introduced into all three subject strains is a [redacted]. It contains [redacted] and [redacted]. It was [redacted]. The purpose of this gene is to [redacted], allowing for [redacted]. The [redacted] gene was first discovered by [redacted] This f[redacted] was isolated from [redacted], and purification and identification of the [redacted] followed ([redacted]). [redacted] belongs to an enzyme class known as the [redacted], which act on [redacted] rather than [redacted]. [redacted] exhibits high specificity toward [redacted] and some of its analogues. [redacted], a ubiquitous [redacted] grows in the form of macroscopic pallets ([redacted]). Strains of [redacted] are used in industrial production for feed and enzyme production.

2. [redacted]

Additional information supplied by the submitter noted that the nucleotide sequence of the [redacted] used in this submission [redacted]) is identical to that obtained by [redacted]) from [redacted] (GenBank accession [redacted]) which cites [redacted] as the source organism.

C. Subject Microorganisms

The taxonomy of the [redacted] recipient strains [redacted] and the three subject [redacted] strains is identical to the parental wild-type strain [redacted] All subject strains are [redacted] strains in which a [redacted] has been introduced [redacted] as a necessary step in strain construction, allowing for [redacted]. The [redacted] was used in all strains, which consists of [redacted]

[redacted]

Apart from the addition of this intergeneric [redacted] and [redacted] from [redacted], some [redacted] is also present in the subject strains. Also, [redacted]. However, these [redacted] do not alter the taxonomy, and the subject strains are still considered to be [redacted]

The addition of the intergeneric genetic material was shown by the submitter to have no discernible phenotypic differences in the three subject strains relative to the recipient strain [redacted]. Various experiments performed by the submitter also indicate that neither [redacted] nor any subject strains are likely to impact primary productivity. Although all subject strains have been selected for improvements in lipid productivity, none show significant changes in biomass productivity, and thus are not expected to have a competitive advantage in the natural environment.

III. ECOLOGICAL INTERACTIONS OF ALGAE IN THE ENVIRONMENT

In this TERA, any or all of SGI's three strains of [redacted]., and perhaps [redacted], will be grown in open ponds with sizes of miniponds, (25 m^2, 75m^2, 0.05 A, 0.1 A , and 1 A). With the outdoor growth of any algal species, there is the potential for the subject strains to be dispersed to nearby surface water bodies or terrestrial environments. Thus, it is important to consider the characteristics of the alga, and its potential interaction with other algae and organisms in the various environmental media into which it may be disseminated.

According to the [redacted] literature review by [redacted]) that was provided by SGI with the TERA [redacted]), there are no records of adverse impacts of the genus [redacted] to any terrestrial plants or animals. There are also no records of toxicity or pathogenicity of [redacted] to any aquatic plants or wildlife, although the potential exists for unanticipated impacts on population-level interspecies competition, and local biogeochemistry. However, strain characterization data and growth and competition experiments suggest the subject strains will behave similarly to the recipient strain.

Algal blooms in water bodies have also been caused by [redacted]., however, they are not considered harmful algal blooms because [redacted] does not produce phycotoxins. This will be discussed in more detail in later sections.

In a broader context, the interactions of algae in aquatic and terrestrial environments and their role in aquatic food webs were discussed in a previous risk assessment for an algal TERA submission by McClung (2017).

A. Aquatic Ecosystems

A number of factors affect the rise and fall of algal populations in the aquatic environment including the physical factors of light, temperature, weather, water movements, flotation, the chemical nutrient status of nitrogen, phosphorus, silicon, calcium, magnesium, potassium, sulfate, chloride, iron, manganese, and other trace elements, and organic matter (Ikawa, 2004). There are a number of biological factors as well including the presence of resting stages, predation, and parasitism. The polyunsaturated fatty acids produced by algae can affect algal growth. In addition, a number of biological substances are known to be produced by algae that inhibit the growth of other algal or of zooplankton grazers, as shown by Pratt (1944; Pratt et al., 1945). Likewise, it has been shown that some algae detect "infochemical" signals from grazers and can change their morphology accordingly to try to avert predation (Lass and Spaak, 2003). Food webs in water bodies are complex and dynamic and have been shown to vary from season to season and with other perturbations of the water body, e.g., eutrophication (Lindeman, 1942; Martinez, 1991).

[redacted]) noted that there are numerous studies on the effects of the genus [redacted] on various

[redacted]

ecological systems, [redacted]

[redacted]

[redacted]

Location	Conclusions	Citation*
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

[redacted]

Aside from occurring in natural water systems, [redacted]

[redacted]

[redacted]

Aquatic Food Webs

Algae and cyanobacteria are the basis of the food web in both freshwater and marine aquatic ecosystems. The phytoplankton community of a typical north-temperate lake has been shown to consist of up to several hundred algal species that co-exist (Kalff and Knoechel, 1978). Phytoplankton diversity is influenced not only by the different ecological niches within a water body (e.g., benthic vs. pelagic regions), but also by a number of temporal and spatial variations in factors such as nutrient supply, temperature, dissolved oxygen, predation, and parasitism (Wehr and Sheath, 2003; Townsend et al.,

[redacted]

1998). Nutrient supply and herbivory are thought to be the most important parameters affecting diversity changes over time. According to Wehr and Sheath (2003), the phytoplankton species composition in lake food web ecosystems is important because the 'functional properties of algal assemblages vary strongly with species composition'. Different taxa are important because features that are sometimes used to classify various species such as photosynthetic pigments, storage products, motility, reproduction, cell ultrastructure, and even DNA sequence have functional importance. For example, nitrogen fixation ability is of great functional importance but is restricted to a limited number of cyanobacteria. Also, photosynthetic pigment production is important, for instance with the red accessory pigment phycoerythrin which has an absorption maximum of 540-560 nm. The presence of this pigment broadens the photosynthetic capacity of an ecosystem by facilitating growth at greater depths (Goodwin, 1974). Autotrophic picoplankton have a strong competitive advantage under phosphorus-limiting conditions (Suttle et al., 1988; Wehr, 1989).

Diversity in the size fractions of phytoplankton is an important aspect of algal communities and thus food webs. For planktonic food webs, cyanobacteria have a dominant role in aquatic productivity. It is these smaller autotrophs that provide excreted dissolved organic compounds that provide substrates for heterotrophic bacterial growth. In addition, cyanobacteria are directly grazed by protozoa (microflagellates and ciliates). This microbially-based food web in which the major portion of autotrophic production occurs is important to the marine food webs. The microbial food web consists of those organisms that are < 1000 µm, and in freshwater benthic ecosystems consists of (presented by increasing size fraction) cyanobacteria and bacteria, followed by microflagellates, diatoms and green algae, which are then consumed by ciliates, rotifers, copepods, oligochaetes, nematodes, and then invertebrate macrofauna followed by the larger vertebrates (Bott, 1996). A complex microbial food web has bacteria and algae at the lowest trophic level, which are then consumed by protozoa and meiofauna. Meiofauna are organisms in the size range of approximately 50 - 1000 µm and includes large ciliates and metazoan (e.g., rotifers, copepods, and oligochaetes).

An important link between microbial food webs and classical food webs are with the autotrophic picoplankton (> 0.2 - 2 µm). These cyanobacteria are grazed mainly by micro-zooplankton (ciliates, flagellates) rather than by cladocerans or copepods (Pernthaler et al., 1996; Hadas et al., 1998). Size affects the sinking rate with smaller planktonic species sinking more slowly. Thus, the smaller species remain more prevalent in the euphotic zone.

[redacted]

B. Terrestrial Ecosystems

[redacted]

Algae occur in nearly all terrestrial environments on earth and are invariably encountered on and beneath soil surfaces (Metting, 1981). Acceptance of algae as bona fide soil microorganisms evolved late in the 19th century when it was recognized that certain groups were restricted to soil, including some in the order Chlorellales (Shihira and Krauss, 1965; Kessler, 1976). [redacted] also reported Chlorophyta [redacted]) were found to dominate sites in north central Florida pine forest after controlled fire and soil sterilization. The recolonization of the sites by these algae was attributed to their airborne nature and was directly correlated with the soil moisture content [redacted]

Over 38 prokaryotic genera and 147 eukaryotic genera have been identified as terrestrial species, the majority of which are truly edaphic (i.e., of, relating to/influenced by soil). As expected solar radiation, water, and temperature are the most important abiotic factors controlling their distribution, metabolism and life histories (Metting, 1981). Biotic interactions are also important, but much less well-understood. Algae play an important role in primary and secondary plant community succession by acting as an integral part of ecosystem. Algal communities living in soil have the principal function of primary production, nitrogen fixation, and stabilization of aggregates (i.e., can prevent soil erosion) (Metting, 1981). Algae concentrations in soils are typically found to be between 10^3 and 10^4 cells/gram but have been reported as high as 10^8 (Metting, 1981).

IV. DISPERSAL OF ALGAE IN THE ENVIRONMENT

As reviewed by Tesson et al. (2016), microalgae have been reported across a wide range of ecosystems, covering almost all latitudes from tropical to polar regions. Due to their relatively small size (few to 500 um), microalgae are dispersed by water, air, and various biotic vectors (e.g., humans and animals) (Kristiansen, 1996; Tesson et al., 2016). These mechanisms and organisms of dispersal were discussed in a previous algal risk assessment by McClung (2017).

A. Dispersal by Water

Passive dispersal of algae by water can occur wherever there is running water between connected water bodies. A study by Atkinson (1988; as cited by Kristiansen, 1996) found that the colonization of a newly constructed reservoir was from the inflow. It was several years later before the appearance of organisms other than those found in the catchment area. Heavy precipitation and flooding can result in algal dispersal by connecting water bodies that are usually isolated. Algal dispersal by water is likely more important in wetter environments that in arid regions.

B. Dispersal by Aerosols

Air is an important dispersal mechanism of algae, and it is thought that algae have spread throughout the globe as aerosols. As early as 1844 Ehrenberg recognized the presence of airborne algae in dust samples collected 300 km off the nearest coast by Darwin in 1939 on the H.M.S. Beagle (as cited by Kristiansen, 1996).

According to a review article by Sharma et al. (2007), "In general, bioaerosols range from 0.02 to 100 µm in diameter and follow the same physical rule as any particle of a similar aerodynamic diameter. They disperse via air movements and settle according to the settling velocity, available impaction, surface, and climatic factors prevailing in the area (Burge and Rogers, 2000). Air movements within a laminar boundary layer surrounding the source usually release such particles. Many of the particles remain in the layer and eventually settle near the source (<100 m), while some are carried aloft with turbulence and transported by the wind over a long distance. The processes responsible for the release and atomization of bioaerosols from natural sources are as follows:

[redacted]

1. Sweeping of the surface or rubbing together of adjacent surfaces by wind and gusts dislodges the bioparticles from the surface. Dried algae caught by the wind are carried away like dust particles (Grönblad, 1933; Folger, 1970).

2. Formation of oceanographic aerosols by wave action and the bursting of bubbles at the water-air interface (Woodcock, 1948; Stevenson and Collier,1962; Maynard, 1968; Schlichting, 1974). Fragments of scums and foams with algal contents along the shoreline of water bodies can be picked up by the wind and carried aloft (Maynard, 1968).

3. During heavy rainfall, algae are splashed up by raindrops and can be entrained into the atmospheric air by thermal winds (Burge and Rogers, 2000).

4. Storm activity over land and sea where great turbulence is experienced.

5. Human activities, such as agricultural practices, construction and maintenance practices, sewage treatment plants (Mahoney, 1968, as cited in Sharma et al., 2007), garbage dumping, highway traffic, and to a limited extent weapons testing and spacecraft launching, can result in the atomization of constituting algae (Schlichting, 1974; Kring, 2000).

6. Atomization of aerosols to a low height also occurs when surface water containing blooms is used for irrigation and recreational activities like boating, jet skiing, and so forth. (Benson et al., 2005)".

Sharma et al. (2007) also stated, based on the result of earlier publications, that green algae, cyanobacteria, diatoms, and tribophytes comprised most of the aero-algae flora. Cyanobacteria dominate the aero-algae flora of tropical regions whereas chlorophytes (green algae) dominate in the temperate regions.

Brown (1964) conducted studies on airborne algae using agar petri dishes suspended in stationary locations in Texas, and impaction studies of algae onto agar petri dishes from moving automobiles in 14 states. He also collected samples from an airplane. The impaction from the moving automobiles and planes yielded the greater numbers and diversity of algae. For example, the agar plates held from a moving car in Pennsylvania yielded 140 algal impactions composed of approximately 25 different genera of algae. A 10-second exposure obtained from a moving car sampling a local dust cloud resulting from plowing of a field recorded 5,000 algal compactions, of which 4,500 were chlorophycean or xanthophycean. [redacted] was among various Chlorophytes found, both in stationary dishes and impaction either by car or plane. The author stated that a large number of different genera and species can be transported in the air. The algal content of dust was quite high at > 3000 cells per m^3. The author concluded that soil is the predominant source of airborne algae.

Schlichting (1969) conducted studies on airborne algae in Michigan and Texas using Millipore filters and bubblers containing soil-water extracts at heights of 6, 15, 30, 75, and 150 feet from the ground. Also, aerial sampling of maritime algae was made from a ship 100 miles off the coast of North Carolina. Over an eight-year period, the number of algae collected never exceeded 8 cells/ft^2. He then estimated that a person at rest would inhale 240 algal cells per hr, which would result in an inhalation exposure of approximately 2880 cells/day. Higher algae numbers were found in the Texas samples from dust than those from water environments.

The diversity and abundance of airborne green algae and cyanobacteria on monuments and stone art works in the Mediterranean Basin was studied [redacted]

[redacted]

In aquatic habitats, microorganisms are known to be concentrated in the surface films and in foams on the water surfaces (Maynard, 1968). Schlichting (1974) conducted studies on the ejection of microorganisms into the air with bursting bubbles. He found that bubbling air through a bacterial culture resulted in 2,000 times more bacteria in the bubble jet droplets. Microorganisms in the range of 0.3 to 30 µm in diameter can be carried in atmospheric water droplets (Woodcock, 1948, as cited by Schlichting, 1974).

Airborne algae are subject to desiccation stress and ultraviolet light exposure (Sharma et al., 2007). Desiccation, the equilibration of an organism to the relative humidity (RH) of the surrounding atmosphere, is an intensive stress that typically, most phototrophic organisms cannot survive (Holzinger and Karsten, 2013). However, there are studies that suggest that some algae can survive desiccation stress (Evans, 1958, 1959; Schlichting, 1961). A comprehensive list of algae capable of surviving desiccation was published in 1972 by Davis. Parker et al. (1969) reported that various cyanobacteria and green algae survived desiccation as viable algae were found in decades-old air-dried soil samples. This is in contrast to Schlichting (1961) who reported survival of only four hours with desiccation stress.

Ehresmann and Hatch (1975) studied the effect of RH on the survival of the unicellular eukaryotic alga *Nannochloropsis atomus* and the prokaryotic alga *Synechococcus* sp. Viable cells of the latter species could be recovered at all the RHs tested (19, 40, 60, 80, and 100%). However, there was a progressive decrease in the number of viable *Synechococcus* cells with lower RHs. There was a stable survival at RH 92% and above. The results with the eukaryotic green alga were very different. No viable cells of *N. atomus* were recovered below 92% relative humidity. In an earlier study Schlichting (1961) found that algae remained viable under a wide range of environmental conditions including RHs of 28-98%. The stress associated with atomization of the algae was responsible for rapid decrease in viability. So perhaps, the gradual air-drying of soil samples as in Parker et al. (1969) did not result in death of the microorganisms.

Recent work by Szyjka et al. (2017) has demonstrated that cultivation of genetically engineered (GE) algae in outdoor ponds can lead to the aerosol release of these organisms. Their data show that algae grown in ponds can travel and be detected in bucket traps as a function of distance and wind direction. Using qPCR to detect both wild-type and the GE strain showed detectable levels in all traps at distances from 5-50 meters away. However, neither strain was able to outcompete local or airborne algae taxa in either the trap buckets or in experiments conducted using local eutrophic and oligotrophic lake water containing local taxa. Their research also showed that airborne algae have high diversity (species detected using ITS2 primers) and can invade any available waters, including members of the species being tested. This only reinforces the conclusion that aerophilous algae can and will travel, both short and possibly long distances when grown in open ponds, and potential risks lie in an alga's ability to survive, establish and persist in the receiving environment. Additionally, the potential for horizontal gene transfer of the GE strains optimized genes is possible, as this same species or close relatives of this species, may be found in the surrounding environment, in both terrestrial and aquatic environments.

[redacted]

The submitter has stated that they are aware of algae dispersal by aerosols and have described various ways to monitor the areas within and surrounding their test site. The results from their monitoring studies from the previous two TERAs (R-19-0001, [redacted] for the green algae *Parachlorella* sp. and for [redacted] will also play a big factor on how they plan to monitor for the current three subject strains.

The known causes of aerosolization at this site for the algae in an open raceway pond are due to paddle-wheel movement, air-CO_2 injection and bubbling, and general splashing at the air-water interface (R-21-0002). The monitoring data for R-19-0001 showed that very low levels in bioaerosols collected near the open raceway ponds. At that time, [redacted] were also cultivated alongside the R-19-0001 *Parachlorella* sp., yet the [redacted] strains were less abundant in air samples even with a greater amount growing than *Parachlorella* sp.

From the [redacted] monitoring data, the submitter reported that very low levels of [redacted] were found from the areas immediately surrounding the 1-acre pond paddlewheels. Molecular methods were also used to monitor [redacted] subject strain [redacted] and none were found in these paddlewheel samples.

Similar to the monitoring of R-19-0001 and [redacted], the proposed methods for [redacted] will also consist of microbiome profiling as well as specific quantification of the subject strains. The goal is to provide insight to both total emissions from the ponds as well as to characterize the ability of the emitted algae to disperse and establish at a distance. Details and discussion of SGI's algae traps and related parameters were provided in the Exposure report for [redacted]

C. Dispersal by Aquatic and Terrestrial Organisms

Aquatic and terrestrial organisms are responsible for algal dispersal. Even fish can act as vectors. For example, numerous species of plankton algae including cyanobacteria, green algae, and diatoms have been found to pass undamaged through the digestive track of the plankton-eating gizzard shad (Velasques, 1939 as cited by Kristiansen, 1996). Insects such as beetles have been found to carry viable algae in their digestive tract (Parsons et al., 1966, as cited by Kristiansen, 1996), and thus, their faecal pellets can distribute algae to new water bodies. Milliger and Schlichting (1968) found 20 species of green algae in the intestinal tract of beetles. Algae dispersal by beetles is a likely mechanism for small water bodies for short distances (Kristiansen, 1996). Other insects can disperse algae to various water bodies. Reville et al. (1967) found that with four species of aquatic Diptera (craneflies and midges), 21 different genera of algae were found on the collected insects. Likewise, Sides (1968) found that the mud dauber wasp was capable of carrying algae and protozoa as nine and four genera, respectively, were isolated from aseptically collected insects. Parsons et al. (1966, as cited by Kristiansen, 1996) reported the presence of 20 genera of viable blue-green algae (currently cyanobacteria), green algae, and euglenoids in and on dragonflies and damselflies. Dragonflies are thought to be able to transport algae possibly long distances (Maguire, 1963).

Water-living mammals and other mammals such as mink, muskrats, and raccoons can transport viable algae on their fur and sometimes in their intestinal tracts. Human activities can also transport algae between water bodies. For instance, the use of felt-soled wading boots has been banned in a number of states as they have been shown to transport non-native larvae, spores, and algae between water bodies. In Vermont, the felt-soled wading boots are believed to have spread didymo, a slimy alga also called rock snot, to various rivers throughout the state. This alga forms dense mats that blanket the bottom of the stream like a shag carpet, changing pristine trout streams to a green, yucky mess, according to

[redacted]

Shawn Good, a fisheries biologist with the state Fish and Wildlife Department (http://usatoday30.usatoday.com/news/nation/environment/2011-04-28-rock-snot-felt-sole-wader-ban_n.htm).

D. Dispersal by Birds

Water birds are the most important vectors for algae dispersal as they can transport live algae on their feet and feathers and sometimes internally in their bills or in their digestive tract. Water birds such as seagulls have been shown to transport algae, particularly aquatic desmids, in wet mud on their feet for long distances (Strøm, 1926 as cited by Kristiansen, 1996). Desiccation is of course of great importance with the viability of live algae transported on the feathers or feet of birds. Algae carried internally in the digestive tract are not subject to desiccation stress.

Migratory birds have a significant role in the transport of algae for long distances. Proctor (1959) studied the carriage of algae in the intestinal tract of numerous migratory bird species obtained from playa lakes in Texas and Oklahoma. A number of freshwater algae species were found in the alimentary canal of 25 different migratory birds. Algae were found in the lower digestive tract of the pied-bill grebe, the green-winged teal, the blue-winged teal, the shoveler, the American coot, the killdeer, the dowitcher, the American avocet, the Wilson's phalarope, and the belted kingfisher. Since many species of blue-green algae (currently cyanobacteria) and green algae do not have spores or specialized resting structures, the algae were assumed to have been transported as vegetative cells. Based upon the rate of movement of the algae through the alimentary tract and the flying speed of some common migratory birds, Proctor (1959) suggested that algae could be easily transferred between lakes 100 - 150 miles apart, with much greater distances possible with cells or colonies in the caecum of the birds.

[redacted] also investigated the transport of algae on and in various waterfowl. He measured the carriage of chlorophyta (green algae), cyanophyta (blue-green algae), chrysophyta (golden algae), euglenophyta, bacteria, fungi, protozoa, and rotifers and on the feet and feathers, and in the bill and gullet, as well as in the faecal matter of 105 birds representing the following 16 species of waterfowl: black duck (*Anas rubripes*), blue goose (*Chen caerulescens*), buffie-head duck (*Bucephala albeola*), Canada goose (*Branta canadensis*), coot (*Fulica americana*), Eastern belted kingfisher (*Megoceryle alcyon*), gadwall *(Anas strepera*), goldeneye (*Glaucinetta clangula americana*), green-winged teal (*Anas carolinensis*), mallard (*Anas platyrhynchos*), redhead duck (*Aythya americana*), ring billed gull (*Larus delawarensis*), ruddy duck (*Oxvura jamaicensis*), spotted sandpiper (*Actitis macularia*), common snipe (*Capella galinago*), and wood duck (*Aix sponsa*).

The field collection experiments demonstrated that the water birds retained viable forms of algae and protozoa both externally and internally. For those organisms carried externally on the feet and feathers, the birds exposed to the air for less than four hours carried a great variety of organisms. Those exposed to air for longer periods of time had fewer viable organisms. With eight hours exposure to air, there were some organisms on the feet of birds, but a greater variety was found to be carried in the bills. The birds exposed to the air longer than eight hours yielded very few organisms. The contents from the gullets sampled produced good algal growth in culture, whereas only a few of the 163 fecal samples contained viable algae or other organisms. Viable organisms found on the waterfowl consisted of 86 species from the feet, 25 species from the feathers, 25 species from the bills, 14 species from the gullets, and 12 organisms from the fecal material.

The following species of green algae were found on the feet of the waterfowl: [redacted]

[redacted]

Although much fewer numbers of green algae, cyanobacteria, golden algae, euglenoids, protozoa, and fungi were found on the bills and faecal material, [redacted] were found on both [redacted]). The green algae found on the bills were [redacted] The green algae found in the faecal material were [redacted]

V. HISTORY OF USE

The genus [redacted] was previously assessed in [redacted] submitted by SGI. It is a member of the [redacted] which is a large algal group including [redacted] algae are found mostly in soil and freshwater. The genus [redacted]

VI. CURRENT USE AND FUTURE USES

SGI and ExxonMobil's ultimate goal is to develop renewable, sustainable, low-carbon, biofuels at world-scale volumes. The research permitted by this TERA is critical to the efforts to reach this goal. The three TERA subject strains of [redacted] are engineered with the [redacted] for environmental tracking.

As previously stated, the purpose for this TERA is to continue to link the biology work in the lab with successful scale-up in the field by experimenting at a manageable scale. Gaining insight into how candidate algae strains perform in industrially-relevant settings will inform the design of the technology and ultimately accelerate its development and deployment. It will also decrease the risk of failure that comes with continuing to design a technology without knowing the conditions and constraints it will ultimately face at-scale.

VII. GENETIC MODIFICATIONS

The genetic modifications done to arrive at the three subject strains are described in detail in the Genetic Construction Report (Cameron, 2021). As described by Cameron (2021), the subject strains were developed using [redacted] Per the TERA, the subject strains were designed, in part, via [redacted] However, the partial data provided for [redacted] in the three subject strains demonstrate that [redacted]. In addition, [redacted]

[redacted] Furthermore, re-sequencing of the three subject strains supports that [redacted] in all three subject strains. However, which [redacted] remains unclear. Of note, for industrial applications, [redacted] is inherently more stable than [redacted] (2009 Flagfeldt, et al.; 2007 Verwaal, et al.). The submitter has observed that over time, similar strains with [redacted] have been genetically stable. A schematic of the genetic modifications as presented in the GCR is below (Cameron, 2021).

[redacted]

[redacted]

[redacted]

According to the submission, the subject strains are [redacted] has been [redacted] as a necessary step in strain construction to allow for the [redacted]. The same [redacted] was used in the construction of all strains, but the [redacted], and hence the identities of the [redacted] differ among the three subject strains.

According to Cameron (2021), [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

The three subject strains have [redacted] using the method outlined above. The genes that were [redacted] are given in the following table.

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

VIII. CONSTRUCT HAZARD ANALYSIS

The potential hazards posed by the genetic modifications and the potential for horizontal gene transfer (HGT) of the introduced genetic material were analyzed by McClung (2021).

A. Introduced Genes

[redacted]

An intergeneric gene introduced into the three subject strains was a [redacted] This [redacted] makes the subject strains [redacted] Thus, there is no concern for HGT of this [redacted] to pathogens in the environment. Hence there is no concern for [redacted] comprising the [redacted] because it is not [redacted].

[redacted]

2. [redacted]

In addition, the [redacted] used in construction of the subject strains [redacted]

[redacted]

[redacted]

[redacted]

According to the submission this [redacted]

Typically, the use of [redacted] in microorganisms intended for the environment is discouraged. However, since the [redacted], it is not a concern. Likewise, the [redacted] is not worrisome in respect to [redacted]

B. Potential for Horizontal Gene Transfer

With environmental introduction of genetically engineered microorganisms, the potential for horizontal gene transfer of introduced genes into other microorganism in the environment warrants consideration. Horizontal gene transfer among bacteria is widespread and is responsible for acquisition of a myriad of traits in bacteria such as antibiotic resistance, xenobiotic degradation pathways, and even pathogenesis. Not nearly as much is known regarding horizontal gene transfer in eukaryotes. It has been thought that the barriers to horizontal gene transfer in bacteria are even worse in eukaryotic organisms because of the complexities in their transcription and translation mechanisms (Raymond and Blankenship, 2003). However, from evolutionary analyses, horizontal gene transfer in eukaryotes is known to have occurred. For example, in evolutionary times, it was a primary endosymbiotic event of a cyanobacterium being engulfed that gave rise to the photosynthetic plastid in the common ancestor of the Plantae, such as red and green algae and higher plants (Chan et al., 2012). Likewise, the mitochondria arose from the endosymbiosis and subsequent genetic integration of an alpha-proteobacterium (Keeling and Palmer, 2008). In addition, investigations of the *Chlorella* genome, specifically *Chlorella variabilis*, suggest the ability for *Chlorella* to produce chitinous cell walls as a result of genetic material uptake from algal viruses, prokaryotes, and fungi (Blanc et al., 2010). Eckardt et al. (2010) hypothesized that the *Chlorella* chitin metabolism genes could have been acquired via horizontal gene transfer from viruses. There are other episodes of lateral gene transfer in eukaryotes, such as the phagocytosis by the sea slug *Elysia chlorotica* of the alga *Vaucheria litorea.* The photosynthetic sea slug maintains the algal plastids which continue to photosynthesize for months within the slug (Rumpho et al., 2008).

Very little is known about horizontal gene transfer from one algal species to another. A search of the literature on horizontal gene transfer in [redacted] did not reveal any studies specifically on horizontal gene transfer in [redacted]. However, there is evolutionary evidence for horizontal gene transfer in algae. Archibald et al. (2003) found that of the 78 plastid-targeted proteins in the chlorarachniophyte alga *Bigelowiella natans*, approximately 21% of them had probably been acquired from other organisms including streptophyte algae, red algae (or algae with red algal endosymbionts), and bacteria. However, in the green alga *Chlamydomonas reinhardtii,* the homologous genes did not show any evidence of

[redacted]

lateral gene transfer. It was suggested that this may be because this green alga is solely autotrophic whereas the *Bigelowiella* is both photosynthetic and phagotrophic.

Qui et al. (2013) hypothesized that Plantae, particularly the red algae, are important in eukaryote genome evolution because of their ability to serve as "sinks" and "sources" of foreign genes through horizontal gene transfer and endosymbiosis, respectively. This hypothesis recognizes the often underappreciated Rhodophyta as major sources of genetic novelty among photosynthetic eukaryotes.

Another instance of potential lateral gene transfer having occurred in algae is the work presented by Raymond and Kim (2012). They found the presence of ice-binding proteins in sea ice diatoms that apparently were essential for their survival in the ice. These protein genes were completely incongruent with algal phylogeny, and the best matches were all bacterial genes. Like bacterial genes, they did not contain introns.

There is one example of horizontal gene transfer from an alga to its DNA virus. By phylogenetic analysis, Monier et al. (2013) demonstrated that the transfer of an entire metabolic pathway, consisting of seven genes involved in the sphingolipid biosynthesis, from the eukaryotic alga *Emiliania huxleyi* and its large DNA virus known as EhV had occurred. Hunsperger et al. (2015) reported the conserved presence of the light-dependent protochlorophyllide oxidoreductases (POR) in four different algal taxa (dinoflagellates, chlorarachniophytes, stramenopiles, and haptophytes). The study concluded that the duplicates of stramenopiles and haptophytes *por* genes are a result of horizontal gene transfer from a Prasinophyte alga.

Turmel et al. (2009) hypothesized that chlorellalean and pedinomonadalean green algae are reduced forms of a distant biflagellate ancestor that might have also given rise to the other known trebouxiophycean lineages. .A more recent study revealed a shared ancestry between the [redacted]

There is no information in the literature on horizontal gene transfer specifically with [redacted] Although from an evolutionary perspective there is evidence that horizontal gene transfer has occurred in green algae, there are no studies that demonstrate horizontal gene transfer with [redacted] or the closely related genera [redacted] Vertical transfer of the introduced genetic material through sexual reproduction to other [redacted] species is also expected to be low since [redacted] is thought to be asexual.

There is no evidence for the presence of plasmids in the recipient [redacted]. PubMed literature searches of titles and abstracts for [redacted] AND plasmid as well as [redacted] AND mobil*, conducted on 29-Jan-2021, returned zero matches. SGI has re-sequenced [redacted] dozens of times and never observed extrachromosomal elements beyond the organellular genomes from the chloroplast and mitochondrion. The absence of extrachromosmal elements in [redacted] suggests that HGT from the subject strains to other microorganisms is likely low.

[redacted] and its derivatives have been a primary focus of SGI's intensive multi-year research program. Over this time, hundreds of strains with [redacted] have been generated. Once transformed, screened, and selected, the program has not observed genetic construct instability. While explicit testing of genetic stability for a given strain/construct over many generations is rarely done, observational evidence suggests that genetic instability is highly unlikely.

[redacted]

IX. POTENTIAL HUMAN HEALTH HAZARDS OF THE RECIPIENT MICROORGANISM SPECIES

The potential human health hazards of the recipient [redacted] strain to the general population and to potentially exposed or susceptible subpopulations have been evaluated (Rahman, 2021).

1. General Population

A. Pathogenicity/Toxicity

There are no reports in the publicly available databases citing any harmful effects of [redacted]. to human health. Members of the genus [redacted] are green algae that are found in very many freshwater lakes. Generally, green algal infections in humans are rare. Two known diseases reported to be caused by members of the green algae are protothecosis and chlorellosis. These are rare pseudofungal diseases in humans and animals caused by the opportunistic algae of the Chlorellaceae family, *Prototheca* spp. (achlorophyllous mutant) and *Chlorella* spp., respectively (Lass-Florl and Mayr, 2007; Pfaller and Diekema, 2004; Hart et al., 2014). Although the genus [redacted] Hence, [redacted] has no relationship with diseases as mentioned above caused by two other species. Therefore, it can be safely say that there are no human health hazards posed by the recipient strain.

[redacted] and its derivatives are not known to produce any toxins. This is true for all Chlorophytes in general. While toxin-producing algae are not a monophyletic taxonomic group, harmful algae are restricted to a few phylogenetic groups. These include eukaryotic algal groupings of haptophytes, dinoflagellates and ochrophytes, as well as cyanobacteria (Hallegraeff, 2004). Toxin production is not associated with green algae. There is no evidence in the available published literature on the toxin production by [redacted]. Hence, it is unlikely that [redacted] would cause any pathogenicity or toxicity effects on general population.

B. Allergenicity

In an article on the health effects of airborne algae and cyanobacteria, it was reported that few airborne microalgae can cause acute health issues including allergy, inflammatory response, hay fever, skin irritation, burning of eyes, rhinitis, sclerosis and respiratory irritation (Genitsaris et al., 2011). Many of these health effects are due to cyanotoxins production by cyanobacteria. However, there are no reports in the literature citing any allergenicity related to [redacted] It is noteworthy that non-genetically modified strains of [redacted] have been grown in open ponds at the Calipatria site for at about four years without causing allergenic reactions in workers.

2. Potentially Exposed and Susceptible Subpopulations

Potentially exposed individuals are workers at the SGI facility. Susceptible subpopulations that warrant consideration differ whether in relation to potential pathogenicity or allergenicity of [redacted] In terms of pathogenicity, susceptible subpopulations would include those whose immune systems are not fully competent such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapies. Susceptible populations for allergenicity concerns are atopic individuals which are those with a genetic predisposition toward developing hypersensitivity reactions to environmental antigens.

A. Pathogenicity/Toxicity

[redacted]

The potential health effects of the recipient strain must be considered for potentially exposed individuals and for susceptible subpopulations. Workers are the potentially exposed subpopulation. However, strains of [redacted] have no history of any pathogenicity or toxicity on potentially exposed subpopulations.

In terms of pathogenicity, susceptible subpopulations include those with not fully competent immune systems such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapy. [redacted] has not been reported as causing any infections in humans. Thus, there is little concern even for those with not fully competent immune systems as they too are routinely exposed to [redacted] algal cells as it is widespread in the environment. Dermal contact of workers to the alga in the open miniponds is not expected as workers will be wearing personal protective equipment required by SGI regulations (e.g., gloves, safety glasses, long pants, and steel-toed shoes) when handling the algae.

In regards to toxicity, there is low concern for potentially exposed or susceptible subpopulations as well as the general population as [redacted] is not known to produce any phycotoxins.

B. Allergenicity

Allergenicity of the microorganism or to the potentially exposed subpopulation, i.e., workers, must be considered. There are no reports on allergenicity caused by [redacted] and therefore, allergenicity to workers should not be a concern. As added precautions, SGI has implemented the use of proper PPE, i.e., gloves, safety glasses, long pants, and steel-toed shoes by the workers to limit the exposure of the agent through various routes including skin contact, eyes, nose and mouth.

In terms of allergenicity, the susceptible subpopulation of atopic individuals, those with a genetic predisposition to develop hypersensitivity reactions to environmental antigens, must be considered. However, atopic individuals are not expected to be working near the ponds and hence are unlikely to be exposed to the microorganisms.

X. POTENTIAL HUMAN HEALTH HAZARDS OF THE SUBJECT MICROORGANISMS

The potential human health hazards of the three subject strains of [redacted] to the general population and to potentially exposed and susceptible subpopulations have been evaluated (Rahman, 2021).

1. General Population

A. Pathogenicity/Toxicity

There are no concerns for pathogenicity/toxicity arising from the introduced genetic material in the subject strains. As described above, [redacted] were introduced into the subject strains. Neither of these genes is associated with virulence or pathogenicity. Thus, the addition of these genes would not be expected to impact [redacted] physiology. Besides, the products of these genes do not pose any concern for toxicity.

Due to the toxic effects on eukaryotic cells, [redacted] has no clinical application in humans ([redacted]. This [redacted]

[redacted]

As neither of these [redacted], there is no concern for [redacted] were to be horizontally transferred to pathogens in the environment. Thus, it is highly unlikely that the subject strains pose any adverse health effects in humans.

B. Allergenicity

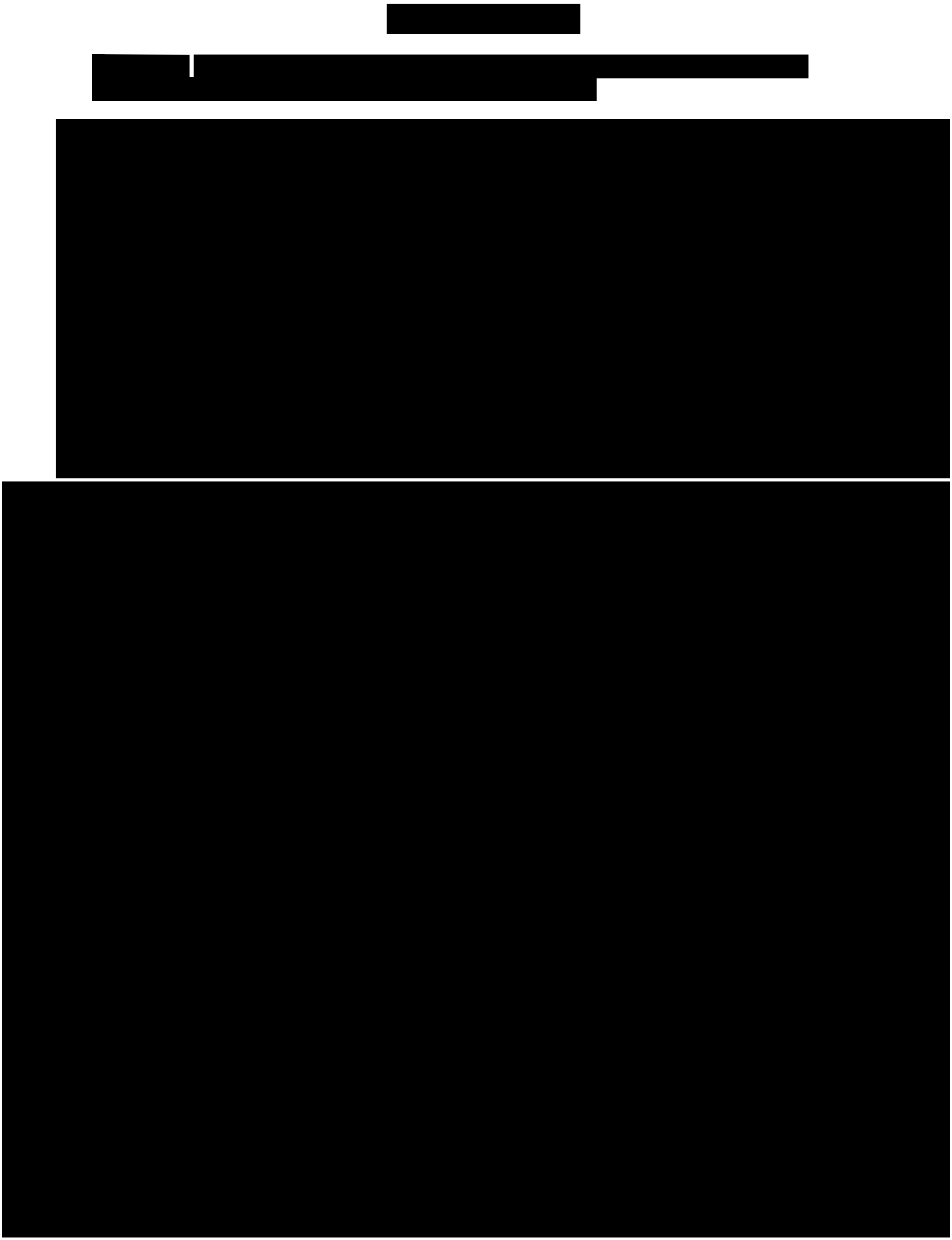
Additions of the intergeneric genes are highly unlikely to cause the subject strains to induce an immunological response in humans. As mentioned above, no reports were found indicating allergic reactions to [redacted]. The protein sequence of [redacted] was used to query the Food Allergy Research and Resource Program "AllergenOnline" database (www.allergenonline.org). As given in the TERA, database queries using the full FASTA amino acid sequence, a sliding 80mer window, as well as exact match for 8mer were conducted. No results above the database thresholds were returned for 80mer or 8mer searches (>35% identity, and 100% identity, respectively). A single hit was returned for the full FASTA search using a conservative threshold e-value of 1. This was for a short (50aa) alignment which has 36% identity (54% similarity) to a [redacted] ([redacted]) from [redacted] A report of allergen cross-reactivity found that sequences less than 50% identical are unlikely to be cross-reactive (Aalberse, 2000). Moreover, the hit identified is for a small segment of the protein and the same author suggests that there is little evidence that short stretches of shared identity lead to allergic cross-reactivity. Similar search with [redacted] encoding protein sequence did not yield any identity with any known allergen. Overall, there is extremely low likelihood of allergenicity for the encoded [redacted].

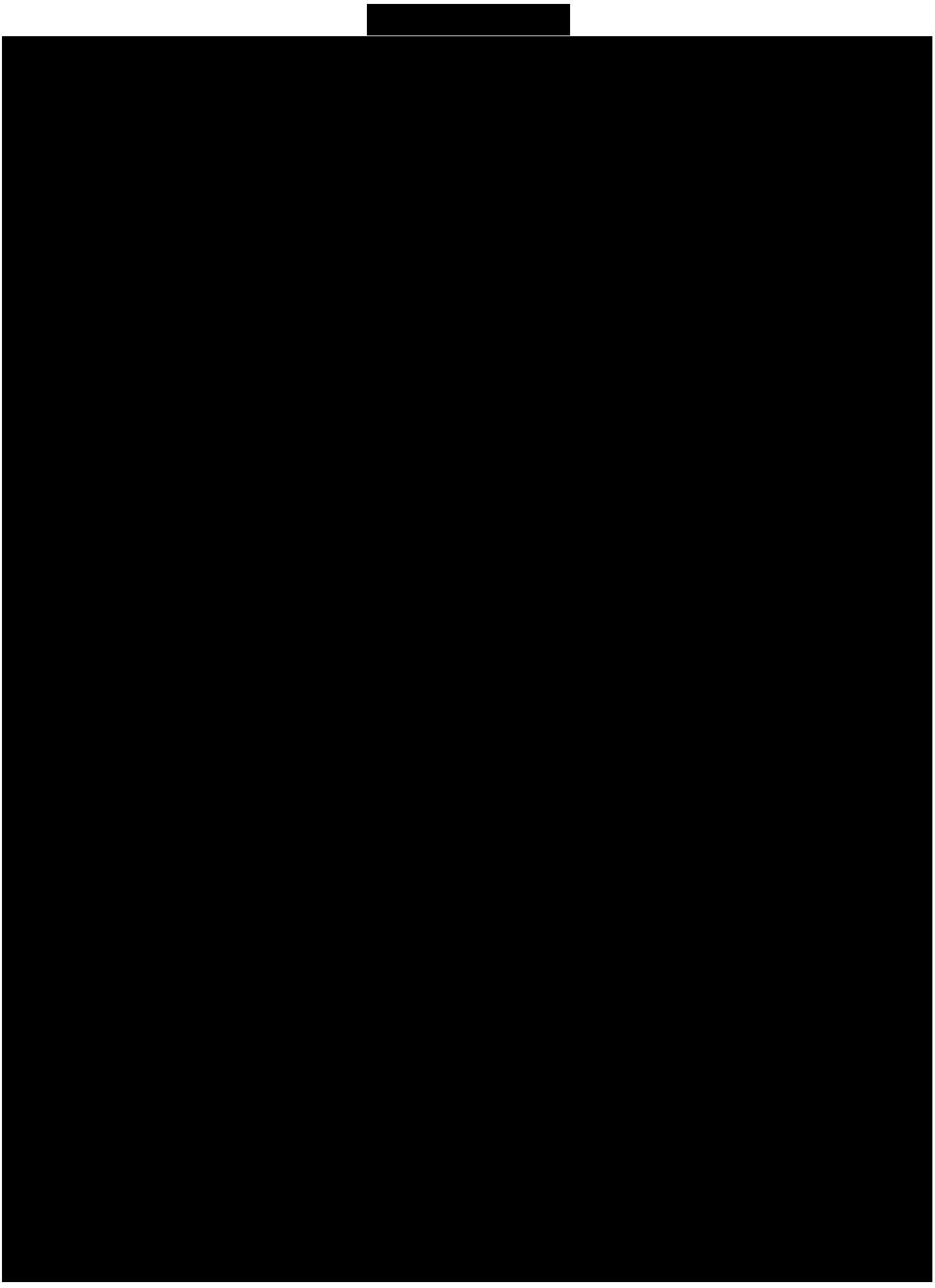
The likelihood of causing any potential allergic reactions by the subject strains will be further eliminated by the use of appropriate PPEs as mentioned above. Movement of cultures between controlled locations onsite at the CAAF is likely to occur through partially-closed transfers that also reduces further exposure of the subject microorganisms.

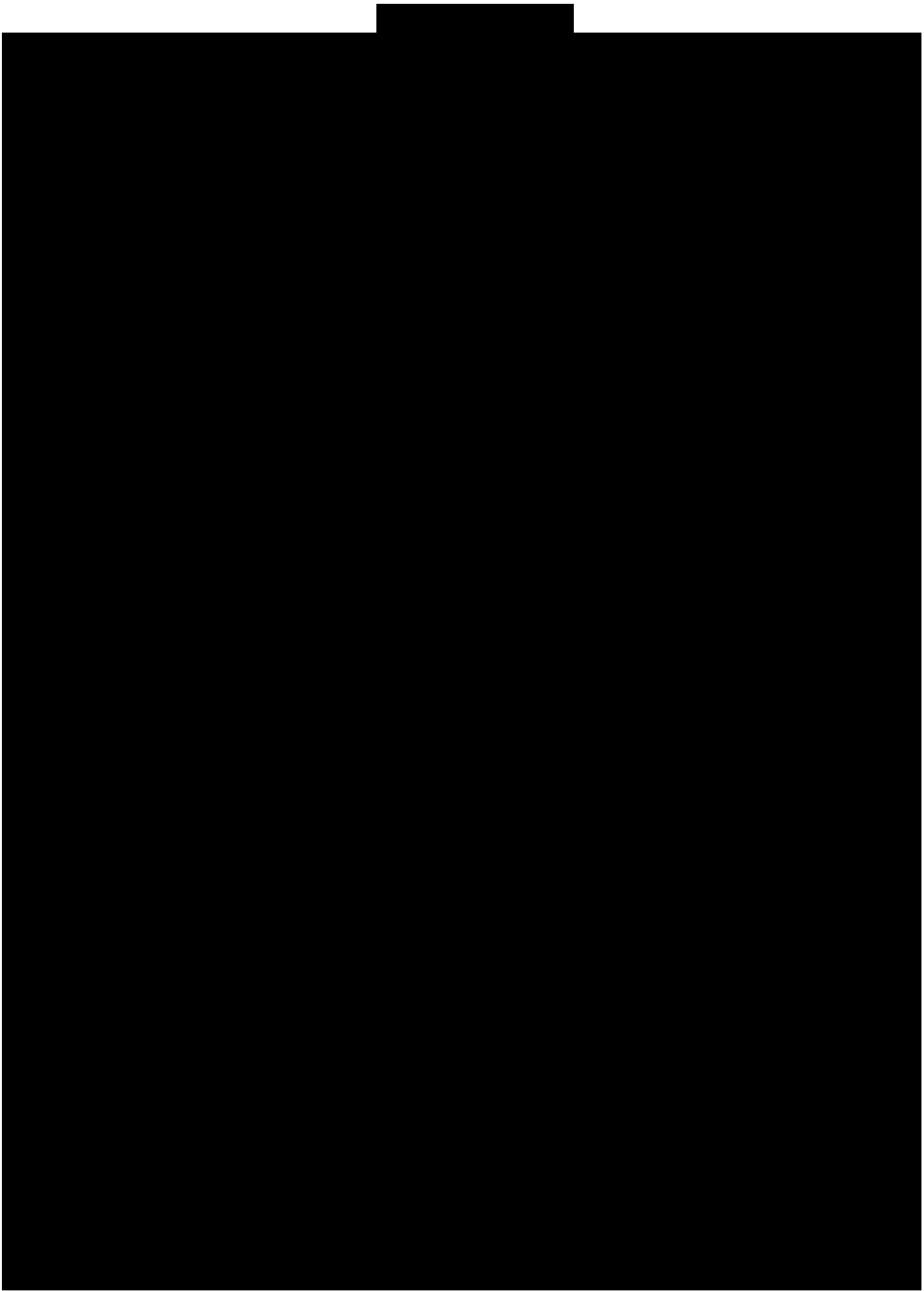
2. Potentially Exposed and Susceptible Subpopulations

The genetic modifications of the recipient to make the three subject strains of [redacted]. do not pose adverse human health effects to potentially exposed and susceptible subpopulations just as they do not to the general human population. The introduced genes encoding for [redacted] do not pose pathogenicity, toxicity, or allergenicity concerns to humans.

XI. POTENTIAL ECOLOGICAL HAZARDS OF THE RECIPIENT MICROORGANISM SPECIES







[redacted]

Although [redacted]

As previously noted, the parental wild-type [redacted] was [redacted] The submitter described that the culture was [redacted], but also grows well in a variety of temperatures and salinities. Classical strain improvement methods [redacted]) were applied to [redacted] to yield the recipient strains [redacted] which were selected for its their marginally increased biomass production and lipid production.

The submission reports that in general, the growth rate of the recipients [redacted] under standard conditions (25 °C, 35 ppt sea salts, pH 8.0, with no added bicarbonate) is somewhat less than one doubling per day, ranging from approximately 0.3x to 0.7x. [redacted] are able to grow under a broad range of abiotic conditions with broad optima centered around [redacted] (Figure 6A), and salinity up to [redacted] (Figure 6B). [redacted] n general have not been observed with flagellated forms or any means of sexual reproduction, as it is only known to reproduce asexually via mitosis.

Figure 6. The recipient strains measured biomass productivity/growth rates under a range of salinities and temperatures. (R-21-0002)

[redacted]

[redacted]

[redacted]

Potential for Algal Blooms by [redacted].

Although some genera in the class [redacted] can cause harmful algal blooms (HABs), the genus [redacted] has not been directly associated with (HABs) as the genus is not listed as a harmful species, including in UNESCO's list of harmful micro algae (webpage: http://www.marinespecies.org/hab/ visited

[redacted]

May 2020). There is also no indication in the literature that [redacted]. produces any toxic compounds. Indirectly, there are reports noting the bloom potential of [redacted] and its role in affecting those water bodies' ecology ([redacted]

Indirectly, there are reports noting the bloom potential of [redacted] and its role in affecting those water bodies' ecology ([redacted]

[redacted] describes [redacted]., among other chlorophytes and cyanobacteria, as main contributors of "massive blooms" in the late summer/fall season. [redacted]

[redacted] who looked into the ecology and taxonomy of phytoplankton in a shallow [redacted] lake, reported that [redacted] (along with *Monoraphidium* sp., *Pediastrum* sp., and *Scenedesmus* sp.) occasionally showed bloom [redacted]

[redacted]

[redacted]

[redacted]

It is also of note that a [redacted]

HAB events can disrupt highly complex stochastic mixing and flushing patterns in freshwaters and increase the eutrophication potential of waterways (Anderson, 2002; Hoagland et al., 2002). Disruptions of these waterways can negatively affect terrestrial wildlife that rely on freshwater ecosystems for food or habitat. As noted above, although there is literature describing the tendency for certain [redacted]. to form blooms, these are often correlated with unnatural water conditions, such as heavy pollution causing a major shift in heavy metal concentrations and pH, which may allow the more tolerant [redacted] to survive and thrive. From the studies listed previously, [redacted] is present in various water bodies worldwide and has shown to be an important part of many complex ecosystems. The existing reports of [redacted] blooms have also been seasonal, indicating its role in succession cycles, co-existing in complicated relationships with various other microorganisms, especially other algae and cyanobacteria.

Potential Effects of [redacted]. on Plants and Animals in Terrestrial Environments

There are no reports in the literature on any adverse effects of [redacted] on plants. Likewise, there are no reports in the literature on animal infections caused by [redacted] There are two other genera of green algae, *Prototheca* and *Chlorella,* that have been shown to cause infections in animals, including humans. The diseases caused by these two genera, protothecosis and chlorellosis, respectively, are very rare but are characterized usually by infection of open wounds. However, as suggested above, HABs if formed can harm terrestrial animals that rely on freshwater sources.

XII. POTENTIAL ECOLOGICAL HAZARDS OF THE SUBJECT MICROORGANISMS

As discussed in the Ecological Hazard Assessment, (Nguyen, 2021), the introduction of the [redacted] and in some cases [redacted], is expected to have no discernible phenotypic differences in any of the three subject strains relative to the recipients [redacted]. The only intended use for [redacted] and [redacted] are as a [redacted] and as a [redacted], respectively, and thus their presence is not expected to introduce any hazard concerns in the three subject [redacted] subject strains.

Various experiments performed by the submitter also indicate that neither recipient nor any subject strains are likely to impact primary productivity. Although all subject strains have been selected for improvements in lipid productivity, none show significant changes in biomass productivity (Figure 7), and thus are not expected to have a competitive advantage in the natural environment.

Figure 7. Characterization of biomass and lipid productivities for all subject strains compared to the recipient strains [redacted] (R-21-0002)

[redacted]

When looking more closely at the biomass composition and lipid profile, the submitter reports that under standard nutrient replete growth conditions the recipient [redacted] From these strain characterization experiments, as well as growth and competition experiments described previously, the results suggest that the subject strains will behave similarly to the recipient strains.

[redacted]

[redacted]

XIII. POTENTIAL SURVIVAL OF THE SUBJECT MICROORGANISMS

As described previously, [redacted] species are distributed worldwide, nearly ubiquitous in regions containing freshwater lakes, streams and rivers as well as in brackish or saltwater sources. In order to assess the potential survival and propagation in the environment of the three subject strains, the submitter tested the ability of the recipient [redacted] s ability to grow in waters collected from water bodies surrounding the test site. Since the intergeneric components [redacted] are not expected to change any phenotypic characteristics in [redacted] the subject strains are expected to perform similarly in these experiments.

Overall, the recipient [redacted] grew to some degree in all the local water types tested, achieving at least one or two doublings with distinct differences between each water sample (Figure 9). The submitter used their algal growth medium as a control to show the full growth potential in optimal conditions (i.e., sufficient nitrogen). It was also concluded that the growth rate correlated with the nitrogen content of the waters at the time of collection.

Along with testing [redacted] survival potential in nearby aquatic receiving environments, several desiccation tolerance studies were performed with soil from the CAAF site (Figure 10). A steep drop in cell viability was observed with [redacted] resulting in a 2 order-of-magnitude reduction in the first 3 days, and no viable cells remained after 8 days (Figure 10B). The submitter reported that in a repeat experiment (not shown), no viable cells were detected after 3 days. An additional desiccation tolerance experiment was conducted using live-dead fluorescence cell stains. [redacted] cells were aliquoted into in sterile 12-well tissue culture plates. Prior to desiccation, cell counts and % viable were determined by flow cytometry. Culture aliquots were set out to dry in a biological safety cabinet. Dryness was achieved after approximately 3 hours. Immediately upon drying and at several timepoints thereafter aliquots were rehydrated with culture media and re-assayed. A small drop in viability is observed immediately after drying and a three log-fold reduction in viable cells is measured after the first 24 hours (Figure 10C).

[redacted]

To complement the experiments in abiotic conditions described above, SGI also conducted similar experiments to test the ability of the recipient strain [redacted] to survive, proliferate and potentially impact the environment in the presence of competition from the endogenous microbiota. Water from two selected SGI stations were passed through a 106 µm stainless steel sieve to remove detritus, larger zooplankton and protists but were not manipulated further in an effort to maintain the native microbiota. Dilutions of [redacted] were added to the samples and various parameters were measured over time. Overall the results indicate that the microbiome of all experimental flasks does change over time. However, there is not a strong partitioning by dose of [redacted] indicating similar microbial communities across all doses.

As discussed in the previous sections, the [redacted] genus encompasses a vast variety of species, able to tolerate a wide range of environmental conditions and stresses. Little research is available that directly shows that [redacted] can survive as well as many other species in the same genera, and more research is required on the SGI isolated strain to determine the true potential for survival posed by the new strain. Ultimately, the survival characteristics are not expected to change from the recipient to the subject strains due to the introduced genetic material or the [redacted]

XIV. DESCRIPTION OF THE FIELD TEST SITE

The field testing of the three [redacted]. subject strains will be carried out at the Synthetic Genomics, Inc. California Advanced Algae Facility (CAAF) in Calipatria, CA. The CAAF is located on private land approximately three miles east of the Salton Sea in the unincorporated area of the County of Imperial, California. The physical address is 250 West Schrimpf Road, Calipatria, CA, 92233. The legal land description is the northwest and southwest quarters of Section 19, Range 14E, Township 11S.

[redacted]

The facility's approximate geographic coordinates are N 33.198491 W 1three.558857. It is bound on the north by McDonald Road and the Imperial Irrigation District's (IID) "O" Lateral and on the south by Schrimpf Road and the IID's "O" Drain. The "O" Lateral is fed by the All American Canal.
Regional access is provided from State Route 111, via McDonald Road. An existing driveway entrance is located on Schrimpf Road. A six-foot chain link fence surrounds the property, with a controlled-access gate on Schrimpf. An east-west six-foot chain link fence divides the property into two forty-acre sections. The northern section is not currently active. The site is staffed with 15-20 full and part time employees. An aerial photo of the CAAF facility is provided below (Figure 11).

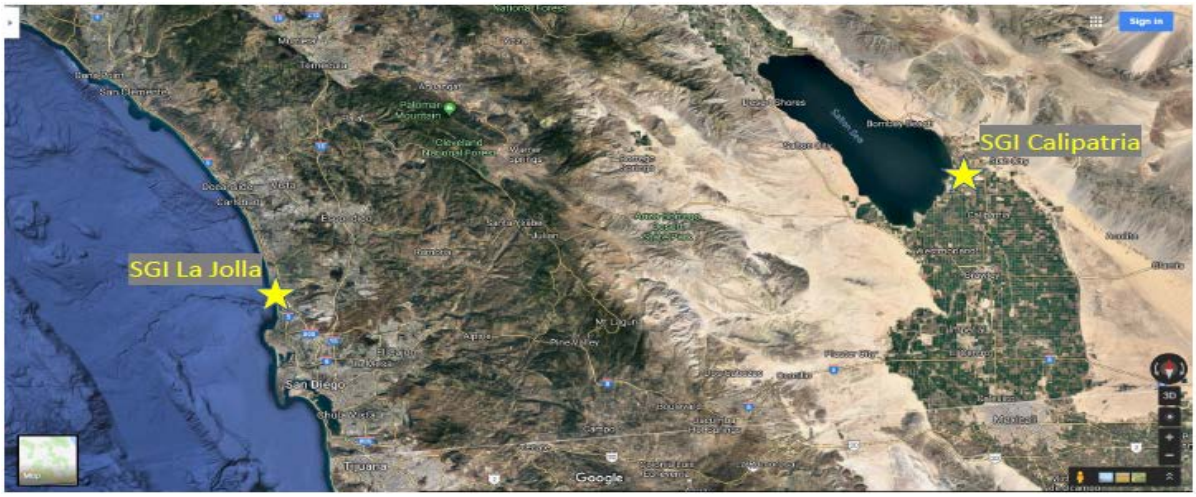
Elevation and slope - The site rests at an elevation of 220 feet below mean sea level, on a plot of land that is exceptionally flat, sloping very gently downward to the west. For reference, the surface of the Salton Sea is approximately 227 feet below mean sea level. A drainage study was commissioned by SGI in 2014.

Proximity to water bodies - The site is located approximately 3 miles from the Southeast corner of the Salton Sea (Figures 13 and 14). The nearest fresh water source (~1.5 miles SSW) is the Alamo River, located to the Southwest of their facility (near sampling location IVF017 in Figure 13). The site draws production water provided by the Imperial Irrigation District (IID),k which sources their water from the Colorado River. The IID transports river water from Yuma AZ, utilizing various open channel irrigation canals that network throughout the Imperial Valley. The site is designated as a zero-discharge facility meaning that none of the water taken onto the site is released back into the local water system (with the exception of rainwater not falling into a pond or collection basin).

Prevailing winds - The prevailing winds are primarily from the southeast. There is a less frequent, but occasional wind pattern with winds coming from due west. Summarized daily averages for one calendar year shows the frequency of winds by quarter.

[redacted]

Figure 12: Satellite image identifying the locations of SGI's R&D Facilities in La Jolla and Calipatria, CA.



[redacted]

XV. STUDIES TO BE CONDUCTED TESTS AT THE FIELD TEST SITE

The primary objective for this experiment is to down-select and cultivate one to three subject strains at a scale of ≥ 25 m^2 (up to 4,000 m^2 or 1.0- A ponds) and maintain production-like operations for up to 18 months. The total duration of this trial is approximately 24 months which includes 6 months of post-cultivation environmental monitoring and for analyses and reporting. An important objective of the proposed test is to work with an engineered alga at a scale which begins to approach the expected scale needed for future commercial viability.

The following tables present all of the various size vessels that will be used, both inside and outside.

[redacted]

For work at all scales, samples will be collected daily for analysis in the CAAF Lab to perform growth measurements as described in Table 7. Briefly, these measurements will include optical density (OD730), AFDW, photosynthetic efficiency (PAM), total organic carbon (TOC), fatty acid methyl ester composition (FAME), microscopic analysis and metagenomic analyses. Excess samples will be disposed of in 0.5% sodium hypochlorite.

The following table presents the types of measurements that will be taken over the course of this TERA and the frequency with which these measurements will be taken.

[redacted]

Table 7. Measurements Conducted and Sampling Frequency.

Measurement	Frequency	Sampling Location
OD_{750}	daily	all raceway ponds, PBRs
AFDW	"	"
Pulse Amplitude Modulation (PAM; Photosynthetic efficiency)	"	"
Lipid content (FAME)	"	"
Total Organic Carbon	"	"
Microscopy	bi-weekly	"
Microbial Ecology	"	raceway ponds
pH	10 minutes	all raceway ponds, PBRs
Water temperature	"	"
Dissolved oxygen	"	"
Conductivity	"	"
Air temperature	"	weather station
Wind speed	"	"
Wind direction	"	"
Photosynthetically active radiation	"	"
Precipitation	"	"
Relative humidity	"	"
Trap pond samples	weekly 1st month; monthly thereafter for ~ 3 months	400 L traps
GE alga in other CAAF ponds	weekly, but also based on non-TERA pond schedules	all raceway ponds
Bioaerosols	weekly 1srt month, monthly for next two, quarterly thereafter	~ 100 m from 403 containment area
Environmental monitoring	monthly	environmental stations at CAAF

XVI. EXPOSURE ASSESSMENT

For a detailed account of potential releases of the production microorganism during laboratory propagation, growth, and waste disposal, see the Engineering Report (Avcin, 2021).

A. Production Volume

The submission provides estimates of the number of microorganisms from PBRs, small ponds (0.1 acre and smaller), and large 1.0 -acre ponds. The submission also provides estimates of [redacted], which the submission states [redacted] the engineering report not include these in the PV estimate.

Approximately [redacted] of algal material may be [redacted]

For the 0.1A and smaller ponds, [redacted] of algal culture may be [redacted] This material may be [redacted].

[redacted]

The total PV was calculated as the [redacted]

[redacted]

B. Process Description

1. Algal Cultivation

The subject microorganisms were created within the labs at SGI where curated cryo-preserved cell banks are maintained. Starting from cryo-preserved cell banks, seed stock cultures are scaled-up indoors in SGI laboratories, dedicated grow rooms, or greenhouses [redacted] (Figure 14). Standard scale-up process [redacted]

[redacted]

The submitter indicates that once the field experiment has been terminated, all biomass in miniponds and PBRs will be inactivated by bleaching the cultures with at least 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal. Biomass in 0.1-acre and 1-acre ponds will be first concentrated within the R&D building. The filtrate water will be treated with bleach and ozone. The concentrated biomass will be retained in a re-circulating tank with at least 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal into the evaporative pond. All equipment will be cleared of the microorganism (including sample containers, ponds, PBRs, etc.) by bleaching or autoclaving and will be discarded as necessary. Any pond spills will be contained within the secondary containment and treated with bleach. The liquid will then be disposed of into the evaporation pond at the CAAF site.

Inactivation studies were performed on [redacted] Experimental data showed that the lowest dose tested (1 mL/L of 4.0% sodium hypochlorite) was sufficient to inactivate [redacted] after one hour (shown in Table I6 of submission). All SGI protocols for inactivation utilize at least 4 mL/L of 12.5% sodium hypochlorite and a minimum contact time of 1 hour to ensure a total deactivated before disposal. Thus, standard SGI CAAF protocols apply greater than a 12.5-fold excess hypochlorite treatment (than that experimentally determined) to inactivate the subject strain providing a conservative treatment for algal cultures.

The submitter also indicated that at the end of the toxicant contact time, the vessels were centrifuged to remove any extracellular toxicant, and the pelleted biomass was utilized to inoculate into fresh media. These cultures were incubated for one week before examining for growth. An inactivation method was deemed to be effective if after one week of growth, no viable cells were observed in the new culture vessels. The technical contact for R19-0001 which used *Parachlorella* sp. stated that they expect 100% inactivation because of the aforementioned SOP, but indicated a minimum of 7-log

[redacted]

inactivation efficiency because the initial culture started at 10^7 CFUs. NCD also assumes a similar log inactivation for this case (consistent with [redacted]), but because the cell concentration is on the order of [redacted], NCD assumes a minimum of [redacted]. The contact report for R19-0001 is attached.

NCD assesses a 100% release scenario. After inactivation, the TERA will be sent to an on-site evaporation pond, and subsequently sent to landfill.

Manufacturing: Laboratory Propagation

Process Description

As previously mentioned, starting from cryopreserved cell banks, seed stock cultures are scaled-up indoors in SGI laboratories, dedicated grow rooms, or greenhouses. For transportation between facilities (i.e., La Jolla and CAAF) cultures are sealed, further contained in secondary spill-proof containers and transported by SGI personnel with enough bleach to neutralize the cultures in the case of a catastrophic failure. The transportation kit will also include nitrile gloves and materials to assist in the cleaning of any spills during transportation. The cultures will only be removed from containers once they reach the inside of the grow room at the CAAF facility.

PROCESSING/USE: Propagation in PBRs and Open Ponds

Days/yr: 52 - 365

[redacted]

Process Description

[redacted]

For work at all scales, samples will be collected daily for analysis in the CAAF Lab to perform growth measurements as described in Table 7. At the end of the experiment, biomass will be [redacted] inactivated using 4 mL/L of 12.5% sodium hypochlorite solution for a contact time of no less than one hour, and discharged to an onsite lined evaporation pond.

[redacted]

Clean-in-place procedures are utilized for cleaning ponds at the CAAF site. At the conclusion of an experiment, ponds are scrubbed along the sides with brushes to remove any films that may have formed over the course of an experiment. Then, ponds are dosed with 4 mL/L of 12.5% sodium hypochlorite and thoroughly mixed with the in-pond paddlewheels. After at least one hour, and after complete mixing, the ponds are then pumped directly to the on-site evaporative disposal pond via a dedicated line.

OCCUPATIONAL EXPOSURE

LABORATORY/GREENHOUSE ACTIVITIES

The submission does not provide worker exposure estimates for laboratory propagation. NCD assumes that some inhalation and dermal exposure in the laboratory / greenhouse setting as a worst case for potential sampling and monitoring growth.

Number of Total Workers: 2

The technical contact for the past case R-19-0001 indicated that typically less than 2 employees are involved in this operation. NCD assumes that this is applicable to this case as the activities occur in the same laboratory and the same activities are performed (consistent with R-19-0001 and [redacted].

Days/yr: 5

The submission does not estimate total exposure days. R-19-0001 assumed 5 exposure days. NCD assumes that this is applicable to this case as the activities occur in the same laboratory and the same activities are performed (consistent with R-19-0001 and [redacted].

Table 8. Summary of the Occupational Exposure Estimates:

Worker Activity	Exposure
Inhalation	
Laboratory/greenhouse activities	[redacted] 2 workers
Dermal	
Laboratory/greenhouse activities	[redacted] 2 workers

PROCESS/USE - Propagation in PBRs and Open Ponds

The submission provided worker estimates in the following table. The submission states “There will be five to six workers involved in the initial inoculation, three to four workers involved in the subsequent activities (e.g., sampling, pond monitoring), and up to 10 workers for harvesting and experimental termination” . Therefore, the workers may perform multiple activities in the table below and NCD estimates the total number of workers to be 20 workers (6 +4 +10 workers).

Table 9. Worker Activity and Duration of Exposure.

[redacted]

Worker Activity	PPE	# of Workers Exposed	Maximum Duration (hr/day)	Maximum Duration (day/yr)
Scale-up of cultures	Safety glasses / disposable nitrile gloves	5-6	10	52
Preparation and pond inoculation		5-6	12	52
Sample collection		3-4	2	365
Pond monitoring		3-4	2	365
Addition of water/ nutrients		3-4	10	365
Harvesting		5-6	10	52
Experimental termination		5-6	10	52
Cleaning ponds	Safety glasses / PVC gloves, rubber boots	8-10	12	52
Lab activities – algae cultivation	Safety glasses / disposable nitrile gloves	3-4	4	365
Lab activities – sample processing		3-4	8	365
Lab activities – general activities		3-4	6	365
Lab activities – analytical testing	Safety glasses / disposable nitrile gloves. Preparation done in hood	3-4	6	365

Number of Total Workers: up to 20

Days/yr: 52 - 365 days/yr (see table above)

PPE: The submission indicates that proper PPE includes gloves, safety glasses, long pants, and steel-toed shoes.

Table 10. Summary of the Occupational Exposure Estimates.

Worker Activity	Exposure
Inhalation	
Sampling of Ponds Near Paddlewheels	[redacted] (365 days/site-yr) 4 workers
Dermal	
Laboratory Work for Daily Sample Processing	[redacted] (365 days/site-yr) 4 workers

Inhalation

1) From: Sampling of Ponds Near Paddlewheels
Up to 4 workers (see table above)
[redacted]/day, 365 days/yr

[redacted]

The submission indicated that weekly aerosol sampling was done for R1-9-0001 and found an average of 22 genome copies/m^3, which is very comparable with the NIOSH study reporting approximately 32-103 CFU/m^3, in a laboratory setting. Similar measurements were conducted for [redacted] for larger raceways ponds and for longer duration. For all 20 samples common airborne microbes were detected and quantified yet the subject strain was not detected. The submission then states for this TERA that "existing EPA estimates should continue to be appropriate, if not slightly overestimated".

As a conservative estimate, NCD references the 1997 Biotech GS area monitoring data collected by NIOSH in a fermentation facility. The GS recommends estimating potential inhalation exposures by taking the most applicable monitoring data and multiplying it by an estimate of the exposure duration. NCD does not have methodology for estimating exposures from aerosolization of liquids near paddlewheels. Therefore, NCD conservatively used data near a centrifuge.

2) From: Inoculation and Scale-Up, PBR sampling, Sample Processing and Experimental Termination

Per the 1997 Biotech GS, potential fugitive air releases from general sampling are shown to be either undetectable or several orders of magnitude lower than other sources. Therefore, compared to other air emission sources (paddlewheels), inhalation exposures from inoculation and scale-up, PBR sampling, sample processing in the laboratory, and experimental termination are considered to be negligible.

Dermal:

1) From: Laboratory Work for Daily Sample Processing
Up to 4 workers (see table above)
[redacted]/day, up to 365 days/yr

The submission indicated that approximately 100 mL of sample will be taken from the pond every day. Per the biotech GS, the potential dermal dose rate is the product of NCD standard dermal exposure assessment factors and the CFU concentration in the appropriate process stream.

ENVIRONMENTAL RELEASE

The environmental releases resulting from both laboratory propagation and from processing/use have been estimated by Avcin (2021).

Laboratory/Greenhouse Activities

Water: negligible

No sources of release to water have been identified other than potential releases from residue in laboratory equipment. NCD's standard assumption for treatment of laboratory-scale equipment (<10 liters) is that releases are negligible. For the CAAF site, the submission states that "All laboratory biological waste is considered hazardous waste and will be disposed of into biological waste containers, then removed from the site and properly managed by a licensed hazardous waste vendor. The site holds both Federal and CAL/EPA registrations." NCD assumes treatment at the La Jolla laboratory is similar.

Air: negligible

NCD's standard assumption is to consider air releases from this activity to be negligible.

[redacted]

Landfill: negligible

No sources of release to this medium have been identified other than potential releases from residue in equipment and PPE.

Incineration: No sources of release to this media have been identified (nor are they typically expected, per NCD generic scenario).

PROCESSING/USE: Propagation in PBRs and Open Ponds

To ensure that the subject microorganisms are completely removed from the test site after the experiment has been completed, all liquid biomass will be treated with 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal. This dose is 12.5-fold greater than the experimentally determined effective dose for killing both recipient and subject strains. Scale-up vessels, including Fernbach flasks and carboys, will be treated with bleach to kill the algal cells before dumping down the drain to the evaporative pond. Carboys will be cleaned and autoclaved for reuse. Biomass from large ponds will be harvested by [redacted] Filtrate water is recycled utilizing additional bleach and ozone treatment. Deactivation of harvested biomass is achieved by treatment in a re-circulating tank with at least 4 mL/L of 12.5% sodium hypochlorite for at least one hour prior to disposal into the evaporative pond. Any additional miscellaneous samples that have been collected from the site will be neutralized by treatment with 4 mL/L of 12.5% sodium hypochlorite for a minimum of one hour before disposal.

Table 11. Summary of the Total Release Estimates to the Environment.

Release	Media	Amount Released
Vessel and Pond Cleaning	Landfill	[redacted]/yr (1 site, 52 days/site-yr) or [redacted]/site-day
Experiment Termination	Landfill	[redacted]/yr (1 site, 52 days/site-yr) or [redacted]/site-day
Bioaerosols	Air	[redacted] CFU/yr (1 site, 365 days/site-yr) or [redacted] CFU/site-day
Total	**All**	[redacted]**/yr**

Water: negligible

The submission states that the CAAF is a zero-discharge site for wastewater. Post inactivation, PBRs, ponds, and harvested material are pumped to an onsite evaporation pond. Evaporated biomass is subsequently sent to landfill.

Air:
[redacted]yr
[redacted]day, over 365 days/yr

Air releases can occur from aerosols generated from agitation due to sparge gas or paddlewheels in the outdoor ponds. Currently, NCD does not have methodology for estimating these types of releases;

[redacted]

therefore NCD estimates this potential release using the methodology described in the Biotech GS for fermentor exhaust gas.

- 52 batches/yr (batches for outdoor ponds are weekly)
- 365 days/yr in ponds (Table G3 and I2)
- [redacted] total volume for outdoor ponds (summation of volume for outdoor ponds from Table G1)
- Aerosolization factor (dimensionless factor indicating the proportion of CFU-containing aerosol particles in the size range of 1 to 10 microns formed per initial number of cells in the liquid volume considered) of [redacted] (NCD GS default)
  [redacted]/mL (final broth concentration)

Bioareosols from fugitive emissions during sampling are considered to be negligible.

Landfill (from evaporation pond):

Per the submission, all process liquid waste is piped to an evaporation pond with a total capacity of 8.6 acre-feet (AF). The pond is permitted by the California Water Quality Control Board Region #7. The pond was designed to comply with Federal, State and County construction standards. Quarterly Reports on the evaporation pond physical integrity, chemical composition and water levels are provided to the State.

Evaporated salt waste material that is >50% water can be shipped via licensed hauler in lined dump trucks to a licensed Class-II landfill for disposal (lined to contain liquids). However, the preferred means of disposal will be to allow the material to dry below 50% water, and when the dried material passes the US EPA paint filter test it will be shipped via a licensed vender in unlined trucks to a licensed Class-III landfill. A Special Waste Profile has been approved by a local landfill.

1) From: Vessel and Pond Cleaning
[redacted]yr
[redacted]day over 52 days/yr

2) From: Experiment Termination
[redacted]/yr CFU/yr
3[redacted]day over 52 days/yr

After growth is complete, the biomass is inactivated and the waste sent to the evaporation pond. NCD assesses 100% release scenario.

5. Consumer, General Population, and Environmental Exposure

The exposures to consumers, the general population, and to the environment were estimated by Townsend (2021).

a. Consumer Exposure

The algal subject strains are not intended for use in consumer products. Therefore, exposure to consumers is not expected.

b. General Population Exposure

[redacted]

For general population exposure, there are two scenarios: Manufacturing/laboratory propagation and Processing/Use.

The Manufacturing scenario did not estimate releases during laboratory or greenhouse propagation. Therefore the following exposure pathways (Air, Water, Landfills, and Incineration) were not assessed.

The Processing/Use scenario estimated releases for the following pathways (Air and Landfills). However, landfill disposal regulations (state and federal), and landfill design are expected to mitigate the exposures to negligible levels. Waste from either direct collection or via solids from the evaporation pond, is sent to landfill that or Class-II or Class-III facility, depending upon the water content, according to applicable Federal and State laws. Releases to water via landfill leaching are not assessed, as exposures are expected to be mitigated by the state regulations. The table below summarizes the estimates for inhalation exposure 100 meters from release source.

[redacted]

Table 12. General Population Exposures.

Summary of General Population Exposures				
Scenario	Activity	Release	Amount Release	Inhalation Exposure (CFU/yr)[a]
Processing/Use	Vessel and Pond Cleaning	Landfill	[redacted] CFU/yr (1 site, 52 days/site-yr) or [redacted] CFU/site-day	Negligible
	Experiment Termination	Landfill	[redacted] CFU/yr (1 site, 52 days/site-yr) or [redacted] CFU/site-day	Negligible
	Bioaerosols	Air	[redacted] CFU/yr (1 site, 365 days/site-yr) or [redacted] CFU/site-day	[redacted] CFU/m^3
Potential for migration to groundwater is possible via landfill leaching, however, it was not quantitatively assessed. There is the potential for bioaerosols emissions from the open ponds.				
[a]Model estimates assume a 100 m receptor distance from release source. [b]Distance from major residential sites was estimated using google satellite images. Processing/use site indicated the closest residences to be approximately 1.5 miles (2,414 m) from the site.				

Land Pathway:

The expected release to landfill from processing/use at the Calipatria, CA site was assessed. Waste will be sent to landfills. The design and operations of these landfills are regulated by the state of California. These engineering controls are expected to mitigate exposures from waste disposed at landfills.

Per the submission, all process liquid waste is piped to an evaporation pond with a total capacity of 8.6 acre-feet (AF). The pond is permitted by the California Water Quality Control Board Region #7. The pond was designed to comply with Federal, State and County construction standards. Quarterly Reports on the evaporation pond physical integrity, chemical composition and water levels are provided to the State. Figure G4 submitted in the Figures and Tables attachment shows the evaporation pond liner detail and the overall liner engineering configuration.

Evaporated salt waste material that is >50% water can shipped via licensed hauler in lined dump trucks to a licensed Class-II landfill for disposal (lined to contain liquids). However, the preferred means of disposal will be to allow the material to dry below 50% water, and when the dried material passes the US EPA paint filter test it will be shipped via a licensed vender in unlined trucks to a licensed Class-III landfill. A Special Waste Profile has been approved by a local landfill.

[redacted]

Vessel and Pond Cleaning

Landfill disposal regulations (state and federal), and landfill design are expected to mitigate the exposures to negligible levels.

Experiment Termination

Landfill disposal regulations (state and federal), and landfill design are expected to mitigate the exposures to negligible levels.

Air Pathway:

Inhalation Exposure

Bioaerosol Fugitive Emissions

To estimate exposures from this source the Gaussian algorithm described in Turner (1970) was used. The standard scenario assumes a release height of 10 meter; a 100 meter receptor distance from the source, a wind speed of 5.5 m/sec and a neutral atmospheric stability. Submission provides wind dispersal information in of wind speeds measured with the highest reported appearing to be 5-6 m/sec, mostly in the direction of southwest and southeast. NCD assumes 5.5 m/sec wind speed.

Using the estimated maximum release of [redacted] CFU/yr-site-strain, the concentration in ambient air 100 meters downwind would be [redacted] CFU/m^3 using standard NCD default models for fugitive releases.

Using NCD standard model, exposures are expected to be low ([redacted] cfu/yr). While submitter data from R-19-0001 indicates some algal dispersal (7 genome copies/m^3 at ~100 m -> which assuming 1 genome copy = 1 CFU gives an estimated inhalation exposure of approximately [redacted] CFU/day using the above equation), this is still within the bounds of the site. This exposure would likely be experienced by workers.

It should be noted that human and animal vectors such as birds could cause dispersal of the microorganisms. In addition, adverse weather could cause further release and dispersal of the microorganisms. The submission notes that "weather for the CAAF is constantly monitored by an on-site weather station and through news media weather forecasts. In the event where highly adverse weather conditions are likely to arise, such as heavy rains or high winds, the site management will make a determination as to whether the inactivation of any ponds or PBRs, or mitigation by other methods, are necessary to minimize the potential loss of primary containment.

Staff is trained to follow an emergency response SOP and to respond to any failures by dosing the area with at least 4 mL/L of 12.5% sodium hypochlorite, before transferring liquids to the evaporation pond. The secondary containment around the [redacted] is designed to hold 5x as much liquid as is contained in the ponds. Barrels of bleach are stored on a dedicated pad within the 403 secondary containment area with a pump adjacent to the secondary containment area that is sufficient to neutralize all of the biomass within the ponds and PBRs".

2. Drinking Water Exposure

No drinking water exposure to the subject microorganisms is expected from the proposed open pond field tests. According to the submission, the CAAF is a zero-release facility where no releases to water

[redacted]

will occur. After inactivation using 4 ml/L of 12.5% sodium hypochlorite (bleach) for at least one hour, all the pond liquid and liquid created by cleaning of PBRs and other equipment is sent to an on-site evaporative pond. After evaporation, the residues in the pond are sent to a landfill.

XVII. INTEGRATED RISK ASSESSMENT

There is low risk associated with the proposed field testing of Synthetic Genomic's three genetically modified strains of the green alga [redacted] in open ponds. The wild-type strain, [redacted], isolated from [redacted] was identified by phylogenetic analysis as belonging to the genus [redacted], however, a species designation was not possible. Thus, the strains are being called [redacted] The recipient strains, [redacted], resulting from [redacted] of the wild-type strain, were selected for their marginally increased biomass production and lipid productivity compared to the wild-type strain.

All three of the algal strains were modified by the introduction of a [redacted] encoding the [redacted] based on the sequence found in the [redacted]

In addition to these introduced genes, [redacted] to increase lipid synthesis. There were [redacted] which included [redacted], [redacted] There was one [redacted], and three [redacted]

There is low risk of injury to human health associated with the outdoor testing of the three intergeneric strain of [redacted]. in open ponds ranging in size up to 1 acre. The subject strains do not present concerns for pathogenicity, toxicity, or allergenicity to humans. Members of the genus are not known to produce phycotoxins. The introduced genes and the [redacted] do nothing to increase the potential for pathogenicity, toxicity, or allergenicity, even with potentially exposed and susceptible subpopulations. Although the subject strains were created using [redacted]. Thus, there is little concern for compromising the [redacted] value of these [redacted] if horizontal gene transfer (HGT) from the subject strains into pathogens were to occur, which is unlikely.

The outdoor field testing of the three subject strains also presents low risk to the environment. This genus of green algae does not present any concerns for pathogenicity or toxicity to animals or plants. Although there are a few reports globally of occasional algal blooms involving this alga genus, blooms typically form in eutrophic waters, and perhaps on a seasonal basis. These are not "harmful algal blooms" (HABs) because [redacted] does not produce phycotoxins. Although algal blooms are not desirable because of the anoxic conditions that can occur, the introduced genetic material does nothing

[redacted]

to increase any propensity for bloom formation.

Of course, with open pond testing, some aerial dispersal of the subject strains to the environment, be it surrounding bodies of fresh water or soils, is to be expected. However, in addition to its [redacted] state, [redacted] can exist as [redacted] which would greatly reduce aerial dispersion due to the heavier weight of the algal colonies versus individual cells. The company has said that the expected dispersal of these subject strains is even less that of the *Parachlorella* sp. strain that was field tested in 2019 in open ponds (R-19-0001). Even if dissemination into the environment were to occur, there would be low concerns for adverse ecological effects resulting from the genetic modifications, i.e., the [redacted] and the [redacted] as neither of those genes pose any environmental hazards.

Even though there may be some exposure to the general population, susceptible subpopulations, and potentially exposed workers, and also to the environment from the growth, harvesting, and aerial dispersal of the algal strains in open pond testing, there is relatively low exposure to workers, the general population, and potentially exposed and susceptible subpopulations, and the environment. As previously mentioned, [redacted] is ubiquitous in the environment so humans are likely frequently exposed. The genetic modifications done to the recipient strains to create these three subject strains do not increase any potential for adverse effects to human health and the environment. Thus, the proposed open pond testing of these genetically modified algal strains poses low risk to human health and the environment.

[redacted]

REFERENCES

Aalberse, R. C. 2000. Structural biology of allergens. J. Allerg. Clin. Immunol. 106:228-38.

[redacted]

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Altschul, S.F., J.C. Wootton, E.M. Gertz, R. Agarwala, R. Morgulis, A.A. Schäffer and Y-K. Yu. 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272:5101-5109.

Anderson, D.M., P.M. Glibert, and J.M. Burkholder. 2002. Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. Estuaries 25:704-726.

[redacted]

Archibald, J.M., M.B. Rogers, M. Toop, K.-I. Ishida, and P.J. Keeling. 2003. Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigelowiella natans.* Proc. Natl. Acad. Sci. 100:7678-83.

[redacted]

[redacted]

Atkinson, K.M. 1988. The initial development of net phytoplankton in cow green reservoir (Upper Teesdale), a new impoundment in northern England, p 30-43. *In:* F.E. Round (ed.), Algae and the Aquatic Environment. Biopress, Bristol.

[redacted]

Baier, T., J. Wichmann, O. Kruse, and K.J. Lauersen. 2018. Intron-containing algal transgenes mediate efficient recombinant gene expression in the green microalga *Chlamydomonas reinhardtii*. Nucleic Acids Research 46:6909-6919.

[redacted]

[redacted]

Benson, J.M., J.A. Hutt, K. Rein, S.E. Boggs, E.B. Barr, and L.E. Fleming. 2005. The toxicity of microcystin in mice following 7 days of inhalation exposure. Toxicon 45:691-698.

[redacted]

[redacted]

Blanc, G., G. Duncan, I. Agarkova, M. Borodovsky, J. Gurnon, A. Kuo, E. Lindquist, S. Lucas, J. Pangilinan, J. Polle, A. Salamov, A. Terry, T. Yamada, D.D. Dunigan, I.V. Grigoriev, J.-M. Claverie, and J.L. Van Etten. 2010. The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. Plant Cell 22:2943–2955.

[redacted]

Bott, T.L. 1996. Algal in microscopic food webs. *In*: R.J. Stevenson, M.L. Bothwell, and R.L. Lowe (eds.). Algal Ecology – Freshwater Benthic Ecosystems, pp. 573-608, Academic Press, San Diego, CA.

[redacted]

Brown, R.M., Jr., D.A. Larson, and H.C. Bold. 1964. Airborne algae: Their abundance and heterogeneity. Sci. 143:583-585.

[redacted]

10.7717/peerj.5884

Cameron, S. 2021. Genetic Construction Report for R-21-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

Chan, C.X., D. Bhattacharya, and A. Reyes-Prieto. 2012. Endosymbiotic and horizontal gene transfer in microbial eukaryotes. Impacts on the tree of life. Mobile Genetic Elements 2:101-5.

Cordy, D. 1973. Chlorellosis in a lamb. Vet. Patho. 10:171-176.

Davis, J.S. 1972. Survival records in the algae, and the survival role of certain algal pigments, fat, and mucilaginous substances. Biologist 54:52-93.

Eckardt, N.A. 2010. The *Chlorella* genome: Big surprises from a small package. The Plant Cell 22: 2924.

Ehresmann, D.W. and M.T. Hatch. 1975. Effect of relative humidity on the survival of airborne unicellular algae. Appl. Microbiol. 29:352-7.

Evans, J.H. 1958. The survival of freshwater algae during dry periods: Part I. An investigation of the algae of five small ponds. J. Ecol. 46:149-67.

Evans, J.H. 1959. The survival of freshwater algae during dry periods: Part II. Drying experiments. J. Ecol. 47:55-71.

Flagfeldt, D.B., V. Siewers, L. Huang, and J. Nielsen. 2009. Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*. Yeast 26:545–551.

Folger, D.W. 1970. Wind transport of land-derived mineral, biogenic and industrial matter over the North Atlantic. Deep Sea Res. 17:337-352.

[redacted]

Goodwin, T. 1974. Carotenoids and biliproteins. Algal Physiology and Biochemistry 10:176-205.

Gressel, J., C. J. B. van der Vlugt, and H.E.N. Bergmans. 2013. Environmental risks of large scale cultivation of microalgae: mitigation of spills. Algal Res. 2: 286–298.

Grönblad, R. 1933. A contribution to the knowledge of sub-aërial desmids. Soc. Sci. Fenn. Comm. Biol. 4:1-7.

Gyles, C. and P. Boerlin 2014. Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. Vet. Pathol. 51:328–340.

[redacted]

Hadas, O., N. Malinsky-Rushansky, R. Pinkas, and T. Cappenberg. 1998. Grazing on autotrophic and heterotrophic picoplankton by ciliates isolated from Lake Kinneret, Israel. J. Plankton Res. 20:1435-1448.

Haenichen, T., E. Facher, G. Wanner, and W. Hermanns. 2002. Cutaneous chlorellosis in a gazelle (*gazella dorcas*). Vet. Pathol. 39:386-389.

Hallegraeff, G. M. 2004. Manual on Harmful Marine Microalgae. UNESCO: Paris. p 799.

Hart J., L. Mooney, I. Arthur, T. J. J. Inglis and R. Murray. 2014. First case of *Chlorella* wound infection in a human in Australia. New Microbe New Infect. 2: 132–133.

[redacted]

Hoagland, P., D.M. Anderson, Y. Kaoru, and A.W. White. 2002. The economic effects of harmful algal blooms in the united states: Estimates, assessment issues, and information needs. Estuaries 25:819-837.

[redacted]

Holzinger, A., and U. Karsten. 2013. Desiccation stress and tolerance in green algae: Consequences for ultrastructure, physiological and molecular mechanisms. Front Plant Sci 4:327.

Ikawa, M. 2004. Algal polyunsaturated fatty acids and effects on plankton ecology and other organisms. UNH Center for Freshwater Biol.Res.6:17-44.

Jitsukawa, K., R. Suizu, and A. Hidano. 1984. Chlorella photosensitization - new phytophotodermatosis. Internat. J. Dermatology 23:263-268.

Kalff, J., and R. Knoechel. 1978. Phytoplankton and their dynamics in oligotrophic and eutrophic lakes. Ann. Rev. Ecol. Systematics 9:475-495.

Keeling, P.J., and J.D. Palmer. 2008. Horizontal gene transfer in eukaryotic evolution. Nature Rev. 9:605-18.

Kessler, E. 1976. Comparative physiology, biochemistry, and the taxonomy of Chlorella (Chlorophyceae). Plant Systematics and Evolution 125:129-138.

[redacted]

Kontoyiannis, D. P., K. A. Marr, B. J. Park, B. D. Alexander, E. J. Anaissie, T. J. Walsh, J. Ito, D. R. Andes, J. W. Baddley, J. M. Brown, L. M. Brumble, A. G. Freifeld, S. Hadley, L. A. Herwaldt, C. A. Kauffman, K. Knapp, G. M. Lyon, V. A. Morrison, G. Papanicolaou, T. F. Patterson, T. M. Perl, M. G. Schuster, R. Walker, K. A. Wannemuehler, J. R. Wingard, T. M. Chiller, and P. G. Pappas. 2010. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin. Infect. Dis. 50:1091–1100.

[redacted]

Kring, D. 2000. Impact events and their effect on the origin, evolution, and distribution of life. GSA Today 10:1-7.

Kristiansen, J. 1996. Dispersal of freshwater algae - a review. Hydrobiologia 336:151-157.

Lass, S., and P. Spaak. 2003. Chemically induced anti-predator defences in plankton: A review. Hydrobiologia 491:221-239.

[redacted]

[redacted]

Le Net, J.-L., M.F. Ahmed, G. Saint-Martin, T. Masson, C. Montoisa, and D.L. Longeart. 1993. Granulomatous enteritis in a dromedary (Cumelus dromedaries). Vet. Pathol. 30:370-3.

[redacted]

Lindeman, R.L. 1942. The trophic-dynamic aspect of ecology. Ecol. 23:399-417.

[redacted]

Lumbreras, V., D.R. Stevens, and S. Purton. 1998. Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. The Plant Journal 14: 441-447.

[redacted]

Maguire, B.J. 1963. The passive dispersal of small aquatic organisms and their colonization of isolated bodies of water. Ecological Monographs 33:161-185.

[redacted]

Mahoney, J.L. 1968. A qualitative survey of the airborne algae, protozoa and bacteria at the denton sewage treatment plant. MSc Thesis. North Texas State University, Denton.

[redacted]

Martinez, N.D. 1991. Artifacts or attributes? Effects of resolution on the Little Rock Lake food web. Ecological Monographs 61:367-392.

Maynard, N.G. 1968. Aquatic foams as an ecological habitat. J.Basic Microbiol. 8:119-126.

[redacted]

[redacted]

McClung, G. 2017. Risk Assessment for R-17-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

McClung, G. 2021. Construct Hazard Analysis for R-21-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

[redacted]

Metting, B. 1981. The systematics and ecology of soil algae. Botanical Rev.47:195-312.

[redacted]

Milliger, L.E. and H.E. Schlichting, Jr. 1968. The passive dispersal of viable algae and protozoa by an aquatic beetle. Trans. Am. Microsc. Soc. 87:443-8.

[redacted]

[redacted]

Monier, A., A. Pagarete, C. de Vargas, M.J. Allen, B. Read, J.-M. Claverie, and H. Ogata. 2009. Horizontal gene transfer of an entire metabolic pathway between a eukaryotic alga and its DNA virus. Genome Res. 19:1441-9.

Monteuuis, G., J.L. Wong, C. G. Bailey, U. Schmitz, and J.E.J. Rasko. 2019. The changing paradigm of intron retention: regulation, ramifications and recipes. Nucleic Acids Res. 47:11497–11513.

[redacted]

Nguyen, K. 2021. Ecological Hazard Assessment for R-21-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

[redacted]

Parker, B.C., N. Schanen, and R. Renner. 1969. Viable soil algae from the herbarium of the Missouri botanical garden. Ann. Mo. Bot. Gard. 56:113-19.

Parsons, W.M., H. Schlichting, and K.W. Stewart. 1966. In-flight transport of algae and protozoa by selected Odonata. Transactions of the American Microscopical Society 85:520-527.

[redacted]

[redacted]

Pernthaler, J., K. Šimek, B. Sattler, A. Schwarzenbacher, J. Bobkova, and R. Psenner. 1996. Short-term changes of protozoan control on autotrophic picoplankton in an oligo-mesotrophic lake. J.Plankton Res. 18:443-462.

Pfaller M. A. and D. J. Diekema. 2004. Unusual fungal and pseudofungal infections of humans. J. Clin. Microbiol. 43:1495–504.

Philbey, A.W., I.J. Links, and G.C. Morrice. 2001. Algal infection in sheep grazing irrigated pasture. Australian Vet. J. 79**:**212-214.

[redacted]

Pratt, R. 1944. Studies on Chlorella vulgaris. Ix. Influence on growth of Chlorella of continuous removal of chlorellin from the culture solution. Amer. J. Botany:418-421.

Pratt, R., J.F. Oneto, and J. Pratt. 1945. Studies on Chlorella vulgaris. X. Influence of the age of the culture on the accumulation of chlorellin. Amer. J. Botany:405-408.

Proctor, V.W. 1959. Dispersal of fresh-water algae by migratory water birds. Science 130:623-624.

Qiu, H. H.S. Yoon, and D. Bhattacharya. 2013. Algal endosymbionts as vectors of horizontal gene transfer in photosynthetic eukaryotes. Frontiers Plant Sci. doi:10.3389/fpls.2013.00366.

Quigley, R.R., K.E. Knowles, and G.C. Johnson. 2009. Disseminated chlorellosis in a dog. Vet. Pathol. 46**:**439-443.

R-17-0002. 2017. TSCA Environmental Release Application (TERA). Office of Pollution and Toxics. U.S. Environmental Protection Agency, Washington, DC.

R-18-0001. 2017. TSCA Environmental Release Application (TERA). Office of Pollution and Toxics. U.S. Environmental Protection Agency, Washington, DC.

R-19-0001. 2019. TSCA Environmental Release Application (TERA). Office of Pollution and Toxics. U.S. Environmental Protection Agency, Washington, DC.

Rahman, M. 2021a. Human Health Hazard Assessment for R-21-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

Rahman, M. 2021b.. Taxonomic Identification Report for R-21-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

Ramirez-Romero, R., L.E. Rodriguez-Tovar, A.M. Nevarez-Garza, and A. Lopez. 2010. *Chlorella* infection in a sheep in mexico and minireview of published reports from humans and domestic animals. Mycopathologia. 169**:**461-466.

[redacted]

Raymond, J.A. and R.E. Blankenship. 2003. Horizontal gene transfer in eukaryotic algal evolution. Proc. Natl. Acad. Sci. 100:7419-20.

Raymond, J.A. and H.J. Kim. 2012. Possible role of horizontal gene transfer in the colonization of sea ice by algae. PLoS ONE. 7(5):e35968.

Rumpho, M.E., J.M. Worfula, J. Leeb, K. Kannana, M.S. Tylerc, D.Bhattacharyad, A. Moustafad, and J.R. Manharte. 2008. Horizontal gene transfer of the algal nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica*. Proc. Natl. Acad. Sci. 105:17867-71.

Sapozhnikov, P. V., Arashkevich, E. G., Ivanishcheva, P. S. 2010. Biodiversity. *In*: , A. G.; Kosarev, A. N., (eds). The Aral Sea Environment. Springer Berlin Heidelberg: Berlin, Heidelberg, pp. 235-282.

Schlichting, H.E, .Jr. 1961. Viable species of algae and protozoa in the atmosphere. Lloydia 24:81-8.

Schlichting, H.E. 1974. Ejection of microalgae into air via bursting bubbles. J.Allergy Clin. Immunol. 53:185-188.

Schlichting, H.E., Jr. 1969. The importance of airborne algae and protozoa. J. Air Pollut. Control Assoc. 19:946-951.

[redacted]

Sharma, N.K., A.K. Rai, S. Singh, and R.M. Brown. 2007. Airborne algae: Their present status and relevance. J. Phycol. 43:615-627.

[redacted]

Shihira, I., and R.W. Krauss. 1965. Chlorella: Physiology and taxonomy of forty-one isolates. Maryland: University of Maryland, p.1-97.

[redacted]

Stevenson, R.E., and A. Collier. 1962. Preliminary observations on the occurence of air-borne marine phytoplankton. Lloydia 25:89-93.

[redacted]

Strøm, K.M. 1926. Norwegian mountain algae: An account of the biology, ecology and distribution of the algae and pelagic invertebrates in the region surrounding the mountain crossing of the Bergen railway. Skrifter, Det Norske Videnskaps-Akademy i Oslo I Mat Naturv Klasse:1-263.

[redacted]

Suttle, C., J. Stockner, K. Shortreed, and P. Harrison. 1988. Time-courses of size-fractionated phosphate uptake: Are larger cells better competitors for pulses of phosphate than smaller cells? Oecologia 74:571-576.

[redacted]

[redacted]

Szyjka, S.J., S. Mandal, N.G. Schoepp, B.M. Tyler, C.B. Yohn, Y.S. Poon, S. Villareal, M.D. Burkart, J.B. Shurin, and S.P. Mayfield. 2017. Evaluation of phenotype stability and ecological risk of a genetically engineered alga in open pond production. Algal Res. 24:378-386.

[redacted]

Tesson, S.V., C.A. Skjoth, T. Santl-Temkiv, and J. Londahl. 2016. Airborne microalgae: Insights, opportunities, and challenges. Appl. Environ. Microbiol. 82:1978-1991.

[redacted]

Townsend, C., H. Hollinshead, and N. Mottl. 2021. Exposure Assessment for R-21-0002. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

Townsend, C.R., R.M. Thompson, A.R. McIntosh, C. Kilroy, E. Edwards, and M.R. Scarsbrook. 1998. Disturbance, resource supply, and food-web architecture in streams. Ecol. Lett. 1:200-9.

[redacted]

Velasques, G.T. 1939. On the viability of algae obtained from the digestive tract of the gizzard shad, Dorosoma cepedianum (le sueur). The American Midland Naturalist 22:376-412.

Verwaal, R., J. Wang, J.-P. Meijnen, H. Visser, G. Sandmann, J.A. van den Berg, and A. J.J. van Ooyen. 2007. High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. 73:4342-4350.

Wattier, R. L., C. C. Dvorak, J. A. Hoffman, et al. 2015. A prospective, international cohort study of invasive mold infections in children. J. Pediatric. Infect. Dis. Soc. 4:313–322.

Wehr, J.D. 1989. Experimental tests of nutrient limitation in freshwater picoplankton. Appl. Environ. Microbiol. 55:1605-1611.

Wehr, J., and R. Sheath. 2003. Freshwater habitats of algae. Freshwater Algae of North America Academic Press, San Diego, CA 308:11-57.

Wikipedia: https://en.wikipedia.org/wiki/Green_algae

Woodcock, A.H. 1948. Note concerning human respiratory irritation associated with high concentrations of plankton and mass mortality of marine organisms. J. Mar. Res. 7:56-62.

Yamagishi, T. 1988. Guide book to photomicrographs of the freshwater algae. Uchida Rokakuho: Tokyo.

Zakia, A., A. Osheik, and M. Halima. 1989. Ovine chlorellosis in the sudan. Vet. Record 125(25):625-6.